Universidade do Minho
Escola de Engenharia

Sónia Carina Morais da Silva

Virulence factors of non-*Candida albicans Candida* species

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Dissertation for PhD degree in Biomedical Engineering

This work was realized under supervision of:
Supervisors:
Professor Joana Azeredo
Professor David Williams
Co-supervisor:
Professor Mariana Henriques

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A espantosa realidade das cousas

É a minha descoberta de todos os dias.

Cada cousa é o que é,

E é difícil explicar quanto isso me alegra,

E quanto isso me basta.

In O guardador de rebanhos

Alberto Caeiro, 1915

Abstract /Sumário

Abstract

Infections caused by *Candida* species (candidosis) have greatly increased over recent years, mainly due to the escalation of the AIDS epidemic, population ageing, increasing number of immunocompromised patients and the more widespread use of indwelling medical devices. *Candida albicans* is the main cause of candidosis, however, non-*Candida albicans Candida* (NCAC) species such as *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis* are now frequently identified as potential human pathogens. This increased incidence of NCAC in human infection can be attributed to improved identification methods but might also be a reflection of the high level of resistance often exhibited by these species to certain antifungal agents.

Candida species pathogenicity is facilitated by a number of virulence factors, most importantly adherence to medical devices and/or host cells, biofilm formation, and secretion of enzymes, such as proteases. Despite intensive research to identify pathogenic factors in fungi, particularly in *C. albicans*, relatively little is known about the virulence determinants of NCAC species. Thus, the work described in this thesis examined several of the most relevant virulence factors (adhesion, biofilm formation ability, tissue colonisation and invasion, and expression of hydrolytic enzymes) of clinical isolates of *C. glabrata*, *C. tropicalis* and *C. parapsilosis* recovered from different body sites (oral cavity and urinary and vaginal tracts).

The study of NCAC biofilms, in terms of formation, structure, matrix composition, and metabolic activity, was the first goal of this research. Total biomass quantification, showed that all NCAC strains were able to form biofilms, although this was less extensive for *C. glabrata* compared to *C. parapsilosis* and *C. tropicalis*. Scanning electron microscopy (SEM) revealed structural differences for biofilms with respect to cell morphology and spatial arrangement. Furthermore, *C. parapsilosis* matrices had large amounts of carbohydrates and low protein. Conversely, matrices extracted from *C. tropicalis* biofilms had low amounts of carbohydrates and protein. Interestingly, the composition of *C. glabrata* biofilm matrices' was high in both protein and carbohydrate contents. It was also evident

that there were intrinsic differences in terms of metabolic activity amongst biofilms of NCAC species, and no correlation was found concerning colony forming units (CFUs) and biofilm metabolic activities determined by XTT reduction.

Another objective of the work described was to study the adhesion and biofilm formation ability of several clinical urinary isolates on to silicone in the presence of artificial urine (AU) and the role of *Candida* surface properties (hydrophobicity and zeta potential) in these events. Silicone colonization by NCAC species in the presence of AU showed that, all urinary isolates were able to adhere to silicone, but in a species and strain dependent manner. However, these differences in adhesion could not be correlated with cell surface properties (hydrophobicity and zeta potential). Moreover, despite the high number of cultivable cells biofilms were not observed and confocal scanning laser microscopy (CLSM) showed an absence of extracellular polymeric material for all strains.

The pathogenesis of *C. parapsilosis* and *C. tropicalis* was investigated using a commercially available reconstituted human oral epithelium (RHOE), in conjunction with CLSM observation and lactate dehydrogenase (LDH) determination. Furthermore, the role of secreted aspartly proteinases (Saps) on invasion and damage of RHOE was evaluated by real-time polymerase chain reaction (PCR). It was possible to observe that all *C. tropicalis* and *C. parapsilosis* strains were able to colonize the tissue, however, this was in a species- and strain-dependent manner. *Candida parapsilosis* revealed low invasiveness after 12 h of infection and extensive damage was evident after 24 h when assessed using histological examination and LDH determination. Moreover, *C. tropicalis* was found to be highly invasive after 12 h of infection, with extensive tissue damage occurring also after 24h. Real time-PCR of *SAP* genes showed that expression was strain dependent for both species. Furthermore, the results suggested that the proteinases were not involved in invasion of RHOE by *C. tropicalis* and *C. parapsilosis*, but indicated a role for these enzymes in tissue damage caused by *C. parapsilosis*.

Finally, single and mixed infection of RHOE by *C. glabrata* or/and *C. albicans* were examined, using CLSM and peptide nucleic probes by *in situ* hybridization (PNA FISH). It was found that the invasiveness of *C. glabrata* strains was enhanced in the presence of *C. albicans*.

In summary, this work underlines both species and strain differences in terms of virulence factors associated with *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. Furthermore, there was clear evidence demonstrating the importance of the use of new techniques including CLSM and molecular analysis tools enabling the elucidation of the mechanisms of virulence. By increasing our knowledge on *Candida* pathogenesis, potential therapeutic targets may well be identified that can be used as adjuvant for new therapies.

Sumário

As infecções por *Candida* espécies (candidoses) têm aumentando drasticamente nos últimos anos, principalmente devido ao aumento da idade da população, ao crescente número de pacientes imunocomprometidos e ao elevado uso de dispositivos médicos. *Candida albicans* é o principal agente causador de candidoses, contudo outras espécies de *Candida* não *albicans* (CNA) têm vindo a ser identificadas como potenciais agentes patogénicos, nomeadamente, *Candida glabrata*, *Candida tropicalis* e *Candida parapsilosis*. Este aumento é também o reflexo da elavada resistência destas espécies a certos antifúngicos. A patogenicidade de *Candida* está relacionada com o elevado número de factores de virulência, sendo os mais relevantes a capacidade de aderir a materiais médicos e/ou células do hospedeiro, a facilidade em formar biofilmes e a produção de enzimas, tais como as aspartil proteases (APs). Apesar, de existirem diversos estudos desenvolvidos com o intuito de identificar factores de virulência em *C. albicans*, pouco se conhece sobre os factores de virulência em espécies de CNA. Assim, este trabalho teve como objectivo principal o estudo dos factores de virulência mais relevantes (adesão, formação de biofilme, colonização e invasão de tecidos, e produção de hidrolases) de vários isolados clínicos de *C. glabrata*, *C. tropicalis* e *C. parapsilosis* provenientes de differentes partes do corpo humano (cavidade oral e tractos vaginal e urinário).

O primeiro objectivo deste trabalho foi estudar a capacidade de estirpes de CNA formarem biofilmes, bem como a sua caracterização em termos de estrutura, composição da matriz e actividade metabólica. A quantificação da biomassa total demonstrou que todas as estirpes em estudo são capazes de formar biofilme, contudo, verificou-se que *C. glabrata* produz menos biofilme comparativamente às estirpes de *C. parapislosis* e *C. tropicalis*. A avaliação por microscopia electrónica de varrimento revelou diferenças significativas estruturais nos biofilmes no que respeita à morfologia das células e à sua disposição espacial. A matriz dos biofilmes de *C. parapsilosis* apresentou elavada quantidade de polissacarídeos e baixa quantidade de proteínas. Contrariamente, as matrizes extraídas dos biofimes de *C. tropicalis* apresentaram reduzidas quantidades de

polissacarídeos e proteínas. Curiosamente, os biofilmes de *C. glabrata* apresentaram elevada quantidade dos dois componentes em estudo. Com este trabalho, foi também possível concluir, que existem differenças intrínsecas em termos de actividade metabólica dos biofilmes em estudo e não foi encontrada correlação entre o número de células viáveis e a actividade celular determinada através da redução do XTT.

Outro objectivo do trabalho aqui descrito consistiu no estudo da adesão e formação de biofilme de isolados urinários de CNA em silicone na presença de urina artificial (UA) e na avaliação do papel das propriedades superficiais (hidrofobiciadde e potencial zeta) das células nestes fenómenos. Estes ensaios demonstraram que todas as estirpes são capazes de aderir ao silicone, contudo com elevada variabilidade dependendo da espécie e da estirpe. Porém, os parâmetros físico-químicos das superfícies celulares avaliados não se correlacionaram com os valores de adesão obtidos. Além disso, apesar da elevada viabilidade celular após 72h, estas estirpes não foram capazes de formar quantidade elevada de biofilme, como confirmado por microscopia de confocal laser (MCL), que mostrou ausência de matriz.

A patogenicidade de *C. tropicalis* e *C. parapsilosis* foi estudada em epitélio oral reconstituído (EOR) por MCL e por quantificação da libertação da enzima lactato desidrogenase. Avaliou-se também o papel das APs na capacidade invasiva e no dano causado ao epitélio por PCR em tempo real. No que respeita à infecção, foi possível verificar que todas as estirpes de *C. tropicalis* e *C. parapsilosis* foram capazes de o colonizar, contudo numa extensão que varia com a espécie e com a estirpe. *Candida parapsilosis* revelou-se pouco invasiva ao final de 12 h de infecção, mas causadora de elavado dano no tecido ao fim de 24 h. Por outro lado, *C. tropicalis* demonstrou ser altamente invasiva ao fim de 12 h e igualmente causadora de elevado dano no epitélio oral após as 24 h de infecção. Os estudos de expressão dos genes das APs, demonstram depender da estirpe para ambas as espécies. Além disso, os resultados obtidos sugerem que as APs não têm um papel activo na invasão do epitélio em *C. tropicalis* e *C. parapsilosis*, mas sugerem envolvimento no dano causado por *C. parapsilosis*.

Por último, estudou-se a infeção simples e mista do EOR com *C. glabrata* e *C. albicans*, através de MCL e diferenciando as duas espécies com sondas de ácidos núcleicos por hibridação *in situ*. A co-infecção de *C. glabrata* com *C. albicans* demostrou, que a invasão de *C. glabrata* é favorecida por *C. albicans*.

Assim, este trabalho mostra existirem diferenças significativas entre espécies e estirpes no que respeita aos factores de virulência de *C. glabrata*, *C. parapsilosis* e *C. tropicalis*. É também realçada a importância do uso de novas metodologias, tais como MCL e biologia molecular que permitam a elucidação e controlo dos mecanismos inerentes aos factores de virulência subjacentes às candidoses. O aumento do conhecimento a respeito da patogenicidade de *Candida* é importante uma vez que poderá permitir a identificação de potenciais alvos terapêuticos e concomitantemente, o desenvolvimento de novas terapias alternativas.

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Nomenclature

Symbols

P-Significance value

g- Gravity

Θ–Water contact angle (^{o})

γ_s^+-Electron acceptor surface tension parameter (mJ/m^2)

γ_s^--Electron donor surface tension parameter (mJ/m^2)

ΔG_{sws}–Total free energy variation between entities of a given surface(s) immersed in water (w) (mJ/m^2)

ζ-Zeta potential (mJ/m^2)

%-Percent

ΔC_T-Threshold cycle

C-Degrees

Abbreviations

3D-Threedimensional

Abs-Absorbance

ACT-Actin

ALS-Agglutinin like sequence gene

Als-Agglutinin like sequence protein

AIDS- Acquire Immune Deficiency Syndrome

ANOVA-Analysis of variance

APS-Aspartil proteases

ATCC-American Type Culture Collection

AU-Artificial urine

BCA-Bicinchoninic Acid

BSA-Bovine Serum Albumin

CAN-*Candida* não *albicans*

cDNA-complementary Deoxyribonucleic Acid

CFU-Colony Forming Units

CHROMagar-Chromogenic media agar

CLSM-Confocal Laser Scanning Microscopy

CV-Crystal violet

CWPs-Cell wall proteins

DNA-Deoxyribonucleic Acid

dNTP-desoxynucleoside triphospahte

ECMM-European Confederation of Medical Mycology

EPA-Epithelial adhesin gene

Epa-Epithelial adhesin protein

FCT-Fundação para a Ciência e Tecnologia

FISH–Fluorescent *in situ* Hybridization

GPI-Glycophosphatidylinositol anchor protein

h-Hour

HIV-Human Immunodeficiency Virus

HLP- Haemolytic like protein gene

LDH-Lactate Dehydrogenase

Log-Logarithm

min-Minute

mRNA- messenger Ribonucleic Acid

NCAC-non-*Candida albicans Candida*

NCBI-National Center for Biotechnology Information

ND-No detected

PBS-Phosphate Buffer Saline

PCR-Polymerase Chain Reaction

Pga 30-Predictor GPI anchored protein 30

PLs-Phospholipases

PMS-Phenazine Methosulfate

PNA–Peptide Nucleic Acid

RHOE-Reconstituted Human Oral Epithelium

RVOE-Reconstituted Human Vaginal Epithelium

RNA-Ribonucleic Acid

rpm-rotation per minute

rRNA-ribosomal Ribonucleic Acid

SAP-Secreted aspartly proteinase gene

Sap-Secreted aspartly proteinase protein

SDA-Sabouraud dextrose agar

SDB-Sabouraud dextrose broth

SD-Standard deviation

SEM-Scanning Electron Microscopy

SPSS-Statistical package for the social sciences

UA-Urina artificial

UTIs-Urinary Tract Infections

U-Units

v-Volume

V-Voltage

w-Weight

XTT-2, 3 bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]2-Htetrazolium hydroxide

YNB-Yeast nitrogen base

YTL-Yeast Traffic Light

Publications within the thesis

Part of the results presented in this thesis has been published elsewhere.

Papers in peer reviewed journals:

Sónia Silva, Melyssa Negri, Mariana Henriques, Rosário Oliveira, David W Williams, Joana Azeredo. Biology, epidemiology and virulence factors of *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis.* Submitted **(Chapter I).**

Sónia Silva, Mariana Henriques, António Martins, Rosário Oliveira, David Williams, Joana Azeredo (2009). Biofilms of Non-*Candida albicans Candida* species: quantification, structure and matrix composition. *Medical Mycology*; 47: 681-689. **(Chapter II).**

Sónia Silva, Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo (2010). *In vitro* biofilm activity of non-*Candida albicans Candida* species. *Current Microbiology* DOI: 10.1007/s00284-010-9649-7 **(Chapter III).**

Sónia Silva, Melyssa Negri, Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo (2010). Silicone colonization by non-*Candida albicans Candida* species in the presence of urine. *Journal of Medical Microbiology* DOI: 10.1099/jmm.0.017517-0 **(Chapter IV)**

Sónia Silva, Mariana Henriques, Rosário Oliveira, Joana Azeredo, Sladjana Malic, Hopper Samuel, David Williams (2009). Characterization of *Candida parapsilosis* infection of an *in vitro* reconstituted human oral epithelium. *European Journal of Oral Sciences*; 117: 669-675. **(Chapter V)**

Sónia Silva, Samuel J Hooper, Mariana Henriques, Rosário Oliveira, Joana Azeredo, David W Williams (2010). The role of secreted aspartyl proteinases in *Candida tropicalis* invasion and damage of oral mucosa. *Clinical Microbiology and infection* DOI: 10.1111/j.1469-0691.2010.03248.x **(Chapter VI)**.

Sónia Silva, Mariana Henriques, Hayes A, Rosário Oliveira, Joana Azeredo, David W Williams (2010). *Candida glabrata* and *Candida albicans* co-infection of an *in vitro* oral epithelium. Submitted **(Chapter VII).**

Melyssa Negri, Vânia Gonçalves, Sónia Silva, Mariana Henriques; Joana Azeredo, Rosário Oliveira (2010). Crystal violet expedite method for quantification of was *Candida* adhesion to epithelial cells. *British Journal of Biomedical Science* (In press).

David W Williams, Tomoari Kuriyama, Sónia Silva, Sladjana Malic, Michael A O Lewis (2010). *Candida* biofilms and oral candidosis: treatment and prevention. *Periodontology 2000*; 53: 1-16.

Melyssa Negri, Sónia Silva, Mariana Henrique, Joana Azeredo, Terezinha Svidzinski, Rosario Oliveira. Simple model for *Candida tropicalis* biofilm *in vitro* studies using flow conditions, urinary catheters and artificial urine. Submitted.

Book chapters

Sónia Silva, Mariana Henriques, Rosário Oliveira, David D Williams, Joana Azeredo (2007). Biofilm formation ability by non-*Candida albicans Candida* species. Biofilms: Coming of Age (Gilbert, P., Allison, D., Brading, M., Pratten, J., Spratt, D., Upton, M., eds.), 33-41. The Biofilm Club, Manchester 2007 (ISBN: 0-9551030-1-0).

Proceedings and abstracts:

Sónia Silva, Samuel J Hooper, Mariana Henriques, Rosário Oliveira, Joana Azeredo, David W Williams. Patterns of colonisation and invasion of oral mucosa by *Candida tropicalis* **Candida and Candidiases 2010 ASM Conference**, Miami USA, 22th-26th March 2010.

Melyssa Negri, Claúdia Botelho, Sónia Silva, Marina Henriques, Joana Azeredo, Rosário Oliveira. *Candida tropicalis* biofilms on catheters: formation and effect on urinary epithelial cells ***Candida*** **and Candidiases 2010 ASM Conference**, Miami USA, 22th-26th March 2010.

Cláudia Botelho, Melyssa Negri, Sónia Silva, Mariana Henriques, Joana Azeredo, Rosário Oliveira. Adhesion of non-*Candida albicans Candida* spp to urinary epithelial cells **Candida and Candidiases 2010 ASM Conference**, Miami USA, 22th-26th March 2010.

Ana Raquel Conde, Sónia Silva, Joana Azeredo, Rosário Oliveira Mariana Henriques. Evaluation of the action of several antifungal agents in *Candida* species biofilms. **Biofilms 2009-5th ASM Conference on Biofilms,** Cacun, Mexico, 15th-19th November 2009.

Sónia Silva, Melyssa Negri, Vânia Goncalves, Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo Adhesion and biofilm formation of non-*Candida albicans Candida* species on silicone in the presence of urine **Biofilms 2009-5th ASM Conference on Biofilms,** Cacun, Mexico, 15th-19th November 2009.

Sónia Silva, Mariana Henriques, Rosário Oliveira, Joana Azeredo, Sladjana Malic, Samuel Hooper, David Williams Characterisation of *Candida parapsilosis* infection of an *in vitro* reconstituted human oral epithelium **Trends in Medical Mycology,** Athens, Greece, 18th-21th October, 2009.

Mariana Henriques, Sónia Silva, Ana Raquel Conde, Joana Azeredo, Rosário Oliveira. Cell wall proteins profiles of non-*Candida albicans Candida* species. **Trends in Medical Mycology,** Athens, Greece, 18th-21th October, 2009.

Sónia Silva, Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo. Biofilm activity of clinical oral isolates of Non-*Candida albicans Candida* species. **Eurobiofilms** Rome, Italy, 2-4th September, 2009.

Sónia Silva, Mariana Henriques, Rosário Oliveira, Joana Azeredo, Sladjana Malic, Samuel Hooper, David Williams. *Candida parapsilosis* infection of an in vitro

reconstituted human oral epithelium. **British Society for Dental Research Scientific Meeting**, Glasgow, Scotland, 1st-4th September, 2009.

Sónia Silva, Vânia Goncalves, Melyssa Negri, Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo. Adhesion and biofilm formation ability by clinical urinary isolates of Non-*Candida albicans Candida* species on silicone. **Biofilm III, 3rd International Conference**, at Munich from 6th to 8th October 2008.

Sónia Silva, Mariana Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo. Comparison of biofilms formed by clinical oral isolates of Non-*Candida albicans Candida s*pecies. **18th European Congress of Clinical Microbiology and Infectious Diseases**. Barcelona, Spain 19-22 April 2008.

Sónia Silva, Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo. *Candida tropicalis* isolates: biofilm composition and architecture. ***Candida* and candidiasis**, **ASM Conference**. New Jersey, USA, 24-28 March 2008.

Sónia Silva, Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo. Differences in activity profiles between biofilm and planktonic cells of Non-*Candida albicans Candida* species. **Biofilm Technologies Conference.** Singapore, January 2008.

Sónia Silva, Mariana Henriques, Rosário Oliveira, Joana Azeredo. Comparison of cell wall proteins profiles from seven *Candida parapsilosis* clinical isolates. **1st European Summer School "Proteomic Basics"**, (Brixen/Bressanone, South Tyrol, Italy, 12-18 August 2007).

Sónia Silva, Mariana Henriques, Rosário Oliveira, David Williams, Joana Azeredo. Biofilm formation ability by non-*Candida albicans Candida* species. **Second FEBS Advanced Lecture Course Human Fungal Pathogens** (La Colle sur Loup, France, 11-17 May 2007), Book of Abstracts, P104B, p. 159.

Scope of the thesis

Candida albicans is regarded as the most prevalent fungi involved in both human colonization and infection (candidosis). Although, most cases of candidosis have been attribute to *C. albicans*, more recently, non-*Candida albicans Candida* (NCAC) species (*Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata*) have been identified as common pathogens. This emergence is often associated to the advent of new medical procedures to treat cancer, the increase in invasive medical procedures, the incidence of HIV, and the widespread use of broad spectrum antibiotics. The pathogenesis of candidosis is facilitated by a number of virulence factors, most importantly adherence to medical devices and/or host cells, biofilm formation, and secretion of enzymes, such as proteases. Despite intensive research to identify pathogenic factors in fungi, particularly in *C. albicans* relatively, little is known about the virulence determinants of NCAC species.

The primary event in *Candida* infection is the adherence of the microorganisms to a host and/or medical device surfaces, often leading to the formation of biofilms. Therefore, an important aim of the present work was to study the biofilm of NCAC species in terms of its formation ability, structure, matrix composition and its metabolic activity. A special focus is given to adhesion and biofilm formation of several clinical urinary isolates onto silicone in presence of artificial urine and the role of cells' surface properties (hydrophobicity and zeta potential) in these events.

Secreted aspartly proteinases (Saps) are considered to be key enzymes that contribute to candidal infection by promoting damage of the host mucosa, thereby facilitating the invasion of the organism into the epithelium. The pathogenesis of mucosal candidosis has been investigated in several studies using animal models, primarily in *C. albicans*. Furthermore, a commercially available reconstituted human oral epithelium (RHOE) has successfully been used to investigate *in vitro*

mechanisms of epithelium invasion by *C. albicans*. Thus, another important goal of this work was the investigation of the pattern of colonization of RHOE by *C. parapsilosis* and *C. tropicalis* and the role of secretion of aspartly proteinases on invasion and tissue damage.

Oral candidosis is a frequent problem, especially in immune compromised patients and recently *C. glabrata* has emerged as important pathogen of the oral mucosa alone or in mixed infections, often with *C. albicans*. So, the last aim of this work was to examine single and mixed infection (*C. glabrata* with *C. albicans*) of RHOE by confocal laser scanning microscopy (CSLM), using *in situ* hybridization with peptide nucleic probes for selective staining both species.

Structure of thesis

This dissertation is divided in eight chapters. **Chapter I** is a general introduction, in which the latest information concerning the biology, epidemiology and virulence factors of the *C. glabrata*, *C. tropicalis* and *C. parapsilosis* is presented. The next seven chapters correspond to different parts of the experimental work performed.

Chapter II initially addresses the study of biofilm formation ability of different clinical isolates of *C. glabrata*, *C. tropicalis* and *C. parapsilosis* and secondly, its characterization in terms of structure and matrix composition (protein and carbohydrate contents).

In **Chapter III** a comparison between planktonic and biofilm cells concerning their metabolic activity is reported.

Chapter IV evaluates the adhesion and biofilm formation abilities of different clinical urinary isolates of NCAC species onto silicone surfaces in the presence of artificial urine and the role of *Candida* cell surface properties (hydrophobicity and zeta potential) in these events.

Chapters V and **VI** address the determination of the pattern of colonization and invasion of a reconstituted human oral epithelium (RHOE) model by *C. parapsilosis* and *C. tropicalis* strains, respectively, and its relation with the secretion of aspartyl proteinases (Saps). A comparison of clinical strains obtained from different body sites was made in terms of colonization, invasion and *SAP* gene expression using RHOE, together with confocal laser scanning microscopy (CLSM) and real-time PCR.

The aim of the work present in **chapter VII** was to assess single and mixed species infections of RHOE with *C. glabrata*/*C. albicans* and ascertain the role of *C. glabrata* in the infection using specifics peptide nucleic acid (PNA) probes by fluorescent *in situ* hybridization (FISH).

Chapter **VIII** offers the major conclusions of the dissertation and the suggestions for future work.

Chapter I

General Introduction

The work presented in this chapter was based on the submitted review paper:

Biology, epidemiology and virulence factors *of Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*.

Sónia Silva, Melyssa Negri, Mariana Henriques, Rosário Oliveira, David W Williams and Joana Azeredo.

Abstract

In recent years there has been a significant increase in the incidence of fungal infections in humans (Lass-Flörl, 2009). These infections may either be superficial, affecting the skin, hair, nails and mucosal membranes, or systemic, involving major body organs (Rüping *et al.,* 2008). A number of factors have been implicated in this increased occurrence of fungal disease, but it is generally accepted that the more widespread provision of new medical practices, such as immunosuppressive therapy, invasive surgical procedures and use of broad spectrum antibiotics are highly significant (Samaranayake *et al.,* 2002; Hargety *et al.,* 2003; Kojic *et al.,* 2004).

Of the fungi regarded as human pathogens, members of the genus *Candida* are the most frequently recovered from fungal infections. The *Candida* genus contains over 150 heterogeneous species (Calderone, 2002) but only a minority of these are implicated in human candidosis. Additionally, it is known that approximately 65% of *Candida* species are unable to grow at a temperature of 37°C, thereby precluding these species from being successful pathogens or indeed commensals of humans (Calderone, 2002).

Of the *Candida* species isolated from humans, *Candida albicans* is the most prevalent in both health and disease (Richards *et al.,* 1999; Sobel *et al.,* 2000; Samaranayake *et al.,* 2002) conditions. However, whilst mycological studies have shown that *C. albicans* represents over 80% of isolates from all forms of human candidosis (Samaranayake *et al.,* 2002), in the last two decades the number of infections due to non-*Candida albicans Candida* (NCAC) species has increased significantly (Harris *et al.,* 1999; Kauffman *et al.,* 2000; Samaranayake *et al.,* 2002; Manzano-Gayosso *et al.,* 2008; Ruan *et al.,* 2008). The apparent increased involvement of NCAC

species in human candidosis may partly be related to improvements in diagnostic methods, such as the use of primary agars with ability to differentiate *Candida* species as well as the introduction of molecular techniques in the routine diagnosis of fungemia (Liguori *et al.,* 2009). However, the high prevalence of NCAC species in disease could also be a reflection of their inherently higher level of antifungal drug resistance (González *et al.,* 2008) compared with *C. albicans*, as this would promote their persistence, possibly to the detriment of *C. albicans* in mixed species infections treated with traditional antifungal agents.

Unfortunately, compared with *C. albicans* there are relatively few investigations examining the virulence factors of NCAC species. This general introduction therefore provides information on the current state of knowledge on the biology, epidemiology and virulence of *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* which are three of the most frequent causes of candidosis after *C. albicans*.

Biology of non-*Candida albicans Candida* species

The genus *Candida* comprises an extremely heterogeneous group of fungal organisms that can all grow as yeasts (Figure I.1), which were originally thought to reproduce only by asexual means. Macroscopically, colonies of *Candida* species on the routinely used Sabouraud dextrose agar (SDA) are cream colored to yellowish. Depending on the species, their texture may be pasty, smooth, glistening or dry, wrinkled and dull. All species produce blastoconidia (Figure I.1 A), which may be round or elongated (Larone, 2002). Moreover, members of the genus may also produce a filamentous type of growth such as true hyphae (Figure I.1 F) or more frequently, pseudohyphae (Figure I.1 C).

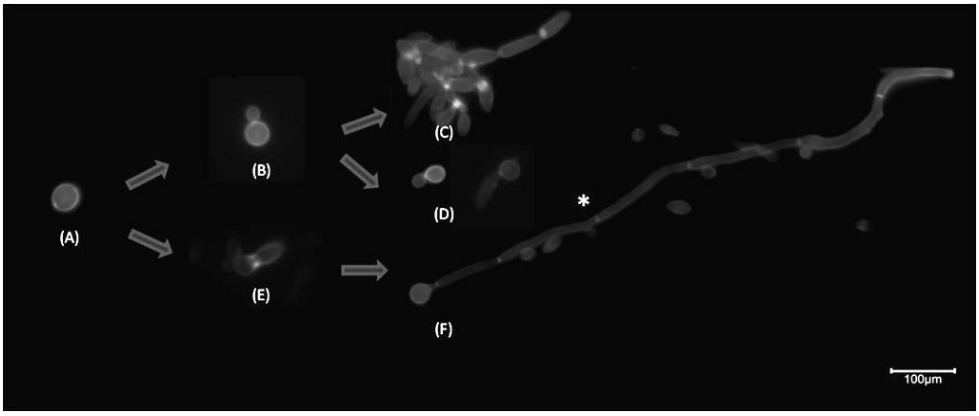

Figure I.1 Epifluorescence photomicrograph of the different morphological growth forms of *Candida albicans* stained with calcofluor white: **A)** blastoconidia; **B)** reproduction by budding; **C)** pseudohyphae formation; **D)** yeast form; **E)** germ tube formation; **F)** hyphae formation. *****Internal cross walls (septa).

The distinction between hyphae and pseudohyphae is related to the way in which they are formed. Pseudohyphae are formed from yeast cells or from hyphae by budding (Figure I.1 B), but the new growth remains

attached to the parent cell and is elongate, resulting in filaments with constrictions at the cell-cell junctions. There is no internal cross walls (septa) associated with pseudohyphae (Figure I.1 C). In comparison, true hyphae are formed from yeast cells or even as branches of existing hyphae. The development of true hyphae is initiated by a 'germ tube' (Figure I.1 E) projection which can elongate and then branch with defined septa that divide the hyphae into separate cells (Figure I.1 F *).

The *Candida* species, *C. albicans* and *C. dubliniensis* are truly polymorphic, due to their ability to form hyphae and/or pseudohyphae, and these particular species are also referred to as being germ tube positive, which is a diagnostic feature (Table I.1) (Calderone, 2002).

Table I.1 Morphological characteristics of *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* species

Species	Germ tube	Hyphae/Pseudohyphae (Dimorphism)	Size of yeasts (µm)	CHROM-agar reaction
C. albicans	+	+/+	4-6 × 6-10	Blue-green
C. tropicalis	-	±/+	4-8 × 5-11	Dark blue
C. parapsilosis	-	-/+	2.5-4 × 2.5-9	White
C. glabrata	-	-/-	1-4	White, Pink-purple

In contrast to other *Candida* species, *C. glabrata* is not polymorphic growing only as blastoconidia (Table I.1; Figure I.2 D). Historically, this species was originally classified in the genus *Torulopsis* due to its lack of pseudohyphae formation. However, in 1978 it was determined that the ability to form pseudohyphae was not a reliable distinguishing factor for members of the genus *Candida* and it was proposed that *T. glabrata* could be classified in the genus *Candida*, because of its human pathogenicity (Fidel *et al.,* 1999). With regards to *C. parapsilosis*, this species does not

produce true hyphae, but can generate pseudohyphae which are characteristically large and curved, and often referred to as 'giant cells'(Figure I.2 E, Larone, 2002; Trofa *et al.,* 2008). In contrast, on cornmeal tween 80 agar and at 25°C after 72 h, *C. tropicalis* can produce, oval blastospores, pseudohyphae and may also produce true hyphae (Figure I.2 F, Larone, 2002; Calderone, 2002; Yoshio *et al.,* 2006).

It should also be highlighted that *C. glabrata* cells (1-4 µm) are noticeably smaller than *C. albicans* (4-6 µm), *C. tropicalis* (4-8 µm), and *C. parapsilosis* (2.5-4 µm) blastoconidia (Calderone, 2002) (Table I.1). On SDA (Figure I.2) *C. glabrata* forms glistening, smooth, and cream-colored colonies, which are relatively indistinguishable from those of other *Candida* species except for their relative size, which can be quite small (Figure I.2 A). Furthermore, *C. parapsilosis* when grown on SDA presents white, creamy, shiny, and smooth/wrinkled colonies (Figure I.2 B). Moreover, on the same medium *C. tropicalis* forms colonies that are cream-colored with a slightly mycelial border (Figure I.2 C, Larone, 2002).

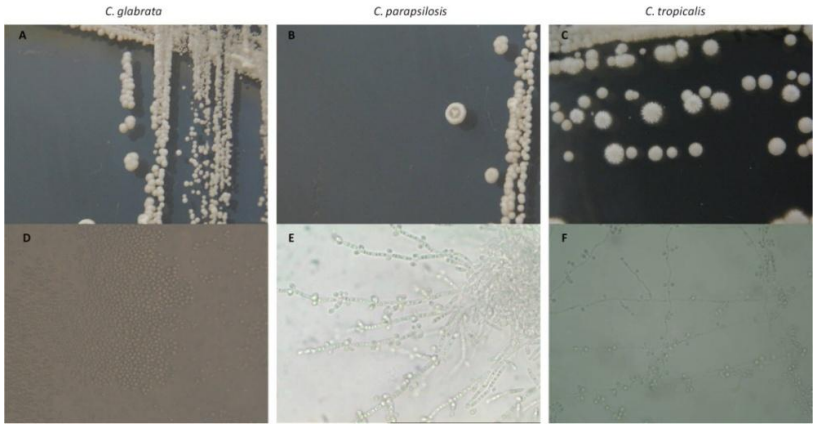

Figure I.2 *Candida* species macroscopic colonies on SDA and microscopy structure on cornmeal tween 80 agar. Macroscopic colonies: *C. glabrata* (**A**); *C. parapsilosis* (**B**); *C. tropicalis* (**C**) and microscopic structure: *C. glabrata* (**D**); *C. parapsilosis* (**E**); *C. tropicalis* (**F**).

On *CHROMagar™ Candida* (CHROMagar, Paris, France), a relatively new differential agar medium, it is possible to distinguish a number of different *Candida* species by colour, as a result of distinct biochemical reactions. On this chromogenic agar, *C. glabrata* colonies appear white, pink to purple, in contrast to *C. albicans* colonies which are blue-green, while *C. parapsilosis* appears white, and *C. tropicalis* dark blue (Table I.1).

Concerning the biochemical reactions of *Candida* species, *C. glabrata* ferments and assimilates only glucose and trehalose, in contrast to *C. albicans*, which ferments and/or assimilates a high number of sugars excluding sucrose (Odds, 1988). *Candida tropicalis* has the ability to ferment and assimilate sucrose and maltose (Martin, 1979). Curiously, *C. parapsilosis* was firstly included as a species of *Monilia,* due to its inability to ferment maltose (Odds, 1988; Trofa *et al,* 2008).

Regarding the genetic aspects of *Candida*, a critical distinguishing characteristic of *C. glabrata* is its haploid genome, in contrast to the diploid genome of *C. albicans* and several other NCAC species (Fidel *et al.,* 1999). Genetically, *C. tropicalis* is most similar to *C. albicans*, and *C. glabrata* the least related (Butler *et al.,* 2009). It is through the advent of molecular genetics, that new identification methods for *Candida* have developed leading to the identification of new species together with their increased recognition in human infection. Therefore, prior to 2005, *C. parapsilosis* was separated into three groups (I to III), and further studies revealed genomic differences that have led to the separation of these groups into closely related but distinct species, namely, *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* (Gácser *et al.,* 2007 a, b).

Epidemiology and risk factors for infection with non-*Candida albicans Candida* species

The high mortality rate associated with bacterial infections has declined with the early administration of empirical antibiotics, whilst concurrently systemic fungal infections have become increasingly important as causes of morbidity and mortality. *Candida* species are amongst the most frequently recovered fungi from blood cultures in hospitalized patients (Pfaller *et al.,* 1998). In fact, an increasing incidence of fungal infections with *Candida* species has been noted in immunocompromised patients, including those patients in intensive care, post-surgical units, and suffering from cancer (Kiehn *et al.,* 1980; Samaranayake *et al.,* 2002; Hargety *et al.,* 2003). *Candida* species are most frequently isolated from the oral cavity, and vulvovaginal and urinary tracts and can be detected in approximately 31 to 55% of healthy individuals (Odds, 1988). Historically, *C. albicans* has accounted for 70-80 % of clinical isolates, while other NCAC species occur only rarely (Odds, 1998). Within the last 5-10 years NCAC species have emerged as opportunistic pathogens of humans and the reasons for this might relate to improved diagnostic methods or altered medical practices, as mentioned above. Regardless of the basis of this change, recent epidemiological data reveal a mycological shift, and whilst *C. albicans* is still the most common causative agent, its incidence is declining with the frequency of other species such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis* increasing (Chandra *et al.,* 2001; Colombo *et al.,* 2003; Basseti *et al.,* 2006).

Pfaller *et al.* (2007), in a study of the epidemiology of invasive candidosis, observed that collectively *C. albicans, C. glabrata, C. tropicalis* and *C. parapsilosis*, accounted for about 95% of identifiable *Candida* infections. Moreover, in the 1980s, according to Kiehn *et al* (1980), *C.*

albicans constituted 68% of *Candida* isolates from sites other than blood in cancer patients, whilst *C. tropicalis*, *C. parapsilosis* and *C. glabrata* only accounted for 12%, 10% and 3.0% of isolates, respectively. Table I.2 highlights the more relevant epidemiologic studies published from 2000 to 2010, concerning oral candidosis, candiduria and candidemia.

Significantly in more recent studies, most cases of fungemia have been associated with NCAC species (Bassetti *et al.*, 2006; Colombo *et al.,* 2007; Hasan *et al.,* 2009; Chakrabarti *et al*., 2009).

The incidence of *C. glabrata* is higher in adults than in children, and is lower in neonates (Krcmery *et al.,* 2002). In particular, *C. parapsilosis* appears to be a significant problem in neonates, transplant recipients and patients receiving parenteral nutrition (Trofa *et al.,* 2008). Furthermore, *C. tropicalis* is commonly associated with patients with neutropenia and malignancy (Colombo *et al.,* 2007).

For many years *C. glabrata* was considered a relatively non-pathogenic saprophyte of the normal flora of healthy individuals and certainly not readily associated with serious infection in humans. However, following the widespread and increased use of immunosuppressive therapy together with broad-spectrum antibiotic therapies, the frequency of mucosal and systemic infections caused by *C. glabrata* has increased significantly (Hajjeh *et al.,* 2004). In the European Confederation of Medical Mycology (ECMM) survey, the incidence rates of candidosis infections attributed to these NCAC species were: 14% to *C. glabrata* and *C. parapsilosis,* and 7% to *C. tropicalis* (Tortorano *et al.,* 2006). Recently, Chen *et al.* (2008) reported that *C. glabrata* was a causative agent of candiduria in Australia. This is extremely important, since, compared with other NCAC species infection, the mortality rate associated with *C. glabrata* is the highest (Abi-Said *et al.,* 1997; Krcmery, 1999).

Table I.2 Epidemiologic studies published during 2000-2010, concerning the distribution of *Candida* species isolates among various types of candidosis

Candidosis	References	Year of observation	Number of studies	Region/ country	Number of strains	*C. albicans* (%)	*C. tropicalis* (%)	*C. parapsilosis* (%)	*C. glabrata* (%)
Oral Candidose	Gravina *et al.,* 2004	2003	62	Venezuela	43	42.3	12.8	14.9	2.1
	Luke *et al.,* 2008	-	101	Argentine	-	60.7	4.5	-	5.6
	Martins *et al.,* 2010	2005-2006	97	Portugal	53	79	4.8	6.5	4.8
Candiduria	Kauffman *et al.,* 2000	-	861	USA	530	51.8	7.9	4.1	15.6
	Dorko *et al.,* 2002	-	-	Slovakia	94	61.7	6.3	24.5	-
	Alvaréz-Lerma *et al.,* 2004	1998-1999	1765	Spain	389	68.4	36	0.5	8.2
	Kobacyashi *et al.,* 2004	-	205	Brazil	45	35.5	22.3	11.1	8.8
	Passos *et al.,* 2005	-	153	Brazil	43	70	4.6	4.6	7
	Binelli *et al.,* 2006	1999-2001	115	Brazil	23	52	43.5	-	17.3
	Chen *et al,* 2008	2006	54	Australia	65	85.2	-	4.4	27.8
Candidemia	Trick *et al.,* 2002	1999	2358	USA	-	59	10	11	12
	Bassetti *et al.,* 2006	1999-2003	182	Italy	182	40	9	23	15
	Tortorano *et al.,* 2006	1997-1999	569	Italy	473	53	6	15	13
	Colombo et al., 2007	-	7038	Brazil	282	38	48	23	9
	Costa-de-Oliveira *et al.,* 2008	2004	117	Portugal	-	35	-	26.5	-
	Hasan *et al.,* 2009	-	51	USA	126	21	38	12	3
	Miranda *et al.,* 2009	2004–2005	63	Brazil	-	42	33	16	2
	Chakrabarti *et al.,* 2009	-	140	India	-	26.3	-	-	10.5

Until recently, few studies had evaluated independent risk factors associated with nosocomial *C. glabrata* acquisition and subsequent infection. Little is known about the hospital reservoirs of *C. glabrata* but, as with *C. albicans*, probable sources include a complex interaction of environmental and human reservoirs. Two studies (Isenberg *et al.,* 1989; Vasquez *et al.,* 1993) have implicated hand carriage on hospital personnel as possible sources of infection. Thus, *C. glabrata* may be similar to *C. albicans* and other nosocomial pathogens that are acquired, directly or indirectly, from contaminated environmental surfaces. However, the role of carriage by personnel in dissemination of *C. glabrata* remains to be clarified. Lately, the most frequent combination of mixed species infection by *Candida* species is *C. glabrata* and *C. albicans*, which has been found in approximately 70% of the patients with oral candidosis (Redding *et al.,* 2002).

Candida parapsilosis, despite being initially considered a non-pathogenic species, was primarily identified as the causative agent of a fatal case of endocarditis in an intravenous drug user in 1940 (Joachim *et al.,* 1940). Furthermore, over the past decade, the incidence of *C. parapsilosis* has dramatically increased. In fact, reports indicate that *C. parapsilosis* is often the second most frequently isolated *Candida* species from blood cultures (Almirante *et al.,* 2006; Colombo *et al.,* 2007; Costa-de-Oliveira *et al.,* 2008). Furthermore, *C. parapsilosis* is one of the fungi most frequently isolated from human hands (Bonassoli *et al.,* 2005) and the second most commonly isolated *Candida* species from normally sterile body sites of hospitalized patients. This species accounts for 15.5% of *Candida* isolates in North America, 16.3% in Europe, and 23.4% in Latin America, outranked only by *C. albicans* (51.5%, 47.8% and 36.5%, respectively) and *C. glabrata* (21.3%) in North America (Messer *et al.,*

2003). However, *C. parapsilosis* fungemia has a lower mortality rate (4%; Kossoff *et al.,* 1998) compared with *C. albicans* and *C. glabrata*.

The increase in the frequency of *C. parapsilosis* infections has been attributed to a variety of risk factors, including the organism's selective growth capabilities in hyper alimentation solutions and its high ability to colonize intravascular devices and prosthetic materials. Additionally, patients requiring prolonged used of a central venous catheter or indwelling device, such as cancer patients, are at an increased risk of infection with *C. parapsilosis*. A recent study (Almirante *et al.,* 2006) of 72 patients in Spain, with invasive *C. parapsilosis*, identified vascular catheterization (97%), prior antibiotic therapy (91%), parental nutrition (54%), prior surgery (46%), prior immunosuppressive therapy (38%), malignancy (27%), transplant receipt (16%), neutropenia (12%), and prior colonization (11%) as risk factors for infections. In a report of 64 episodes of *C. parapsilosis* candidemia from Brazilian hospitals, between 2002 and 2003, the primary risk factors were neutropenia, the use of central venous catheters, and cancer chemotherapy (Brito *et al.,* 2006). The population at greatest risk for nosocomial infection with *C. parapsilosis* is that of extremely low birth weight neonates (Solomon *et al.,* 1984; Voss *et al.,* 1994). In fact, colonization of the skin or gastrointestinal tract is frequently a first step in the pathogenesis of invasive candidal disease, and neonates are especially prone to such infection given their compromised skin integrity, susceptibility to gastrointestinal tract infection, long term need for central venous catheter, and prolonged endotraccheal intubation (Benjamin *et al.,* 2000). Furthermore, *C. parapsilosis* has been isolated from approximately one-third of neonates with gastrointestinal colonization by *Candida* species (Saimain *et al.,* 2001) and from oropharynges of 23% of healthy neonates (Contreras *et al.,* 1994).

Candida tropicalis is one of the three most commonly isolated NCAC species (Álvarez-Lerma *et al.,* 2004; Binelli *et al.,* 2006; Colombo *et al.,* 2007; Hasan *et al.,* 2009). Usually, *C. tropicalis* is considered the third most frequently isolated NCAC species from blood and urine cultures (Kauffman *et al.,* 2000; Álvarez-Lerma *et al.,* 2004). Moreover, in a recent epidemiology study conducted in 12 Brazilian medical centers, *C. tropicalis* was the second most often recovered *Candida* species, accounting for 33-48% of all candidemia cases (Colombo *et al.,* 2007; Miranda *et al*, 2009). Additionally, *C. tropicalis* is often found in patients admitted to intensive care units, especially in patients requiring prolonged catheterization, receiving broad-spectrum antibiotics, or with cancer (Kauffman *et al.,* 2000; Rho *et al.,* 2004; Colombo *et al.,* 2007; Nucci *et al.,* 2007). Furthermore, *C. tropicalis* appears to display a higher potential for dissemination in the neutropenic host than *C. albicans* and other NCAC species. According to Kontoyiannis *et al.* (2001), there are distinct differences in presentation and risk factors of *C. tropicalis* and *C. albicans* fungemia, with the former more persistent and leading to longer intensive care units stays during the course of their infection. This may imply a higher virulence of *C. tropicalis* when compared with *C. albicans*. In fact, some epidemiologic studies (Krcmery *et al.,*1999; Kontoyiannis *et al.,* 2001; Eggimann *et al.,* 2003; Colombo *et al.,* 2007) documented that *C. tropicalis* was associated with higher mortality than other NCAC species and *C. albicans*. This propensity of *C. tropicalis* for dissemination and the high mortality associated can be related to the virulence factors that this species exhibits (Krcmery *et al.,* 2002; Negri *et al.,* 2010a).

Virulence factors of non-*Candida albicans Candida* species

The pathogenesis of invasive candidosis is facilitated by a number of virulence factors, including the ability to adhere to medical devices and/or host cells, biofilm formation, dimorphism, and secretion of hydrolytic enzymes such as proteases, phospholipases and lipases, and haemolysins. Despite, the intensive research to identify pathogenic factors in fungi, particularly in *C. albicans*, relatively little is known about the virulence determinants of NCAC species. This issue is a major limitation in our ability to diagnose, treat, and prevent NCAC species infection.

Adherence ability of non-*Candida albicans Candida* species

The primary event in *Candida* infection is its adherence to host and/or medical device surfaces often leading to the formation of biofilms (Figure I.3 A; Crump *et al.,* 2000; Chandra *et al.,* 2001).

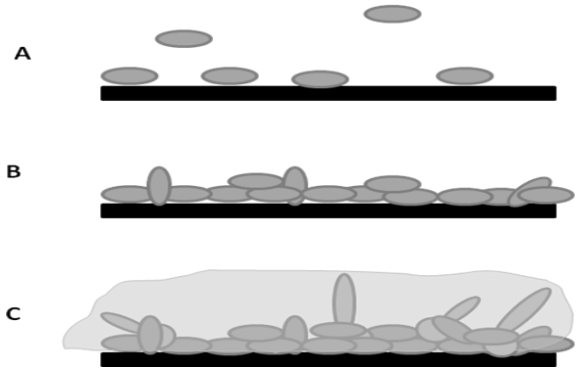

Figure I.3 Stages of biofilm formation of *Candida* species on medical device surface. (**A**) Initial adhesion to surface; (**B**) Formation of basal microcolony layers of *Candida* cells; (**C**) Mature biofilm of *Candida* constituted by cells and extracellular matrix.

Thus, adhesion is an extremely important virulence factor, and the extent of adhesion extension may be dependent on surface properties of both microorganisms and abiotic material, such as hydrophobicity, zeta potential, and surface roughness (Cai *et al.,* 1994; Anil *et al.,* 2001; Henriques *et al.,* 2002; Sousa *et al.,* 2009). Furthermore, the switching between different colony morphologies is associated with alteration in cell wall architecture which in turn can influence cell-to-cell, cell-to-surface, and cell-to-host tissue adherence (Chaffin *et al.,* 2008).

As mentioned previously, the relatively non-pathogenic nature of *C. glabrata* in animal models suggests that it has few virulence attributes. However, its high mortality rate and rapid dissemination would indicate the opposite. In a study with limited number of *C. glabrata* isolates, this organism displayed a degree of hydrophobicity comparable to *C. albicans* (Hazen *et al.,* 1986). Interestingly, while the hydrophobicity of *C. albicans* was extremely sensitive to specific growth conditions, numerous isolates of *C. glabrata* were relatively insensitive to those same growth conditions (Kikutaní *et al.,* 1992). In addition, Camancho *et al.* (2007) did not find a correlation between the hydrophobicity and adherence for *Candida* cells on siliconised latex catheters, demonstrating that cell hydrophobicity alone was not a predictor for the adhesion phenomenon. *Candida glabrata* has been demonstrated to have a two-fold greater tendency to adhere to denture acrylic surfaces compared to *C. albicans* (Luo *et al.,* 2002), and it was again shown that *C. glabrata* adhered to a higher extent to urinary epithelial cells than other NCAC species (Negri *et al.* 2010). In contrast, in an *in vitro* assay of adherence to vascular endothelium, *C. albicans* was the most adherent species, while *C. glabrata* was the least adherent, followed by *C. parapsilosis* and *C. tropicalis* (Klotz *et al.,* 1985). Using a reconstituted human epithelium, a new model for studying microorganism's colonization, Schaller *et al.* (2006) showed that *C. glabrata* was able to colonize but

unable to invade this epithelium. An important factor correlated with the adhesion ability of *Candida* species is the presence of specific proteins on its cell-wall, namely adhesins. In *C. glabrata,* a major group of adhesins is encoded by *EPA* (epithelial adhesin) gene family (De Las Penas *et al.,* 2003). The overall structure of Epa proteins is similar to that of the Als (Agglutinin like sequence) proteins of *C. albicans*. Although, there are few studies concerning *C. glabrata* Epa proteins, it is known that Epa1p is a Ca^{2+} dependent lectin that binds to N–acetyl lactosamine–containing glycol-coconjugates (Comarck *et al.,* 1999). Furthermore, despite the large number of *EPA* genes, it has been shown that deletion of merely *EPA1* reduces adherence *in vitro* (De las Penas *et al.,* 2003). However, although *EPA6* is not expressed *in vitro*, its expression increases during *in vivo* urinary infection, suggesting that *C. glabrata* is capable to adapt to different environmental conditions (Domergue *et al.,* 2005).

As reported for *C. glabrata*, Panagoda *et al.* (2001) shown that the initial adhesion of *C. parapsilosis* cells was associated with surface hydrophobicity. In terms of epithelial and endothelial adhesion ability, few studies have been undertaken with *C. parapsilosis*. The first study (Panagoda *et al.,* 2001) comparing *C. albicans* and *C. parapsilosis* adhesion reported a greater ability (21%) of *C. parapsilosis* for buccal epithelial cell adherence with an increase of 14% in the extent of adhesion to acrylic. These studies suggest that the prevalence and severity of infection caused by *C. parapsilosis* associated with medical devices and/or host surfaces can be due to their high ability to colonize and survive on biomaterial surfaces even in unfavorable conditions. Furthermore, a bioinformatics search of pathogen-specific gene families of *Candida* species revealed a number of genes for cell wall proteins in *C. parapsilosis*. This study included genes for 5 Als proteins and 6 Pga 30 (predicted GPI anchored protein 30) (Butler *et*

al., 2009), but unfortunately there has been no further work in studying the roles these proteins play in *C. parapsilosis* adhesion.

Candida tropicalis has the ability to colonize urinary epithelial cells, silicone and latex catheter (Negri *et al.,* submitted), however the extent of adhesion is strain dependent. Concerning, proteins from *C. tropicalis* cell wall, at least 3 *ALS* genes were identified through southern and western blotting analysis with anti-Als antibody (Hoyer *et al.,* 2001) however, to the authors' knowledge no further work has been undertaken in this area.

Apart from *C. albicans*, our knowledge of adhesion mechanisms in medical devices and human cells pathogenic fungi is still limited. However, the cell wall is thought to play a crucial role for colonization and infection, and therefore, elucidation of its structure and composition would lead to a better understanding of the pathogenesis of NCAC infections, and also to an improvement of their treatment since some cell wall components may constitute valuable targets for antifungal drugs.

Biofilm formation ability by non-*Candida albicans Candida* species

Initial attachment of *Candida* to host or/and medical devices is followed by cell division, proliferation (Figure I.3 B), and subsequently biofilm development (Figure I.3 C) (Ramage *et al.,* 2006). Biofilms are described as surface associated communities of microorganisms embedded within an extracellular matrix. It is now considered that biofilms represent the most prevalent growth forms of microorganisms (Al-Fattani *et al.,* 2006).

Biofilm formation is a potent virulence factor for a number of *Candida* species, as it confers significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and protecting

cells from host immune responses (Donlan *et al.,* 2002; Mukherjee *et al.,* 2004). Moreover, biofilms formed by *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* isolates have been associated with higher mortality rates compared with isolates incapable of forming biofilms (Kumamoto*,* 2002). *Candida albicans* biofilm formation is associated with the dimorphic switch from yeast to hyphal growth, and the biofilm structure involves, generally, two distinct layers: a thin, basal yeast layer and a thicker, less compact hyphal layer (Donlan *et al.,* 2002). It is assumed that the formation of mature biofilms and subsequent production of extracellular matrix is strongly dependent upon species, strain, and environmental conditions (pH, medium composition, oxygen) (Ramage *et al.,* 2006; Jain *et al.,* 2007). Little is known about the matrix composition of NCAC species biofilms, but according to Baillie *et al.* (2000), *C. albicans* biofilm matrix is mainly composed of carbohydrate, protein, phosphorus, and hexosamines. Many previous studies have focused on *C. albicans* biofilms, due its well-recognized virulence, whereas only few reports of NCAC species biofilms are available. Shin *et al.* (2002) showed that biofilm formation by *C. glabrata* was reduced when compared with other NCAC species, when grown in rich culture media. While extensive work has been performed concerning the *C. albicans* genes involved on biofilm formation, little is known about equivalent controlling genes in *C. glabrata*.

Biofilms are readily formed by *C. parapsilosis* cells grown in media containing high glucose and lipid concentrations, which can be associated with the increased prevalence of bloodstream infections in patients receiving parental nutrition. The selective preference of this species for plastic medical devices is of particular interest, as biofilm formation enhances the capacity of the organism to colonize catheters and intravascular cellular lines (Weems, 1992; Trofa *et al.,* 2008). In contrast to *C. albicans*, *C. parapsilosis* biofilms are thinner, less structured, and consist

exclusively of aggregate blastospores (Kuhn *et al.,* 2002). It is known that biofilms are affected by farnesol, a quorum-sensing molecule produced by *C. albicans*. Martins *et al.* (2010) have reported that *C. parapsilosis* cells secrete farnesol, a finding that was in contrast to earlier studies by Trofa *et al.* (2008). However, according to the latter, treatment with extracellular farnesol did arrest growth without an apparent effect on *C. parapsilosis* morphology. Two recent studies documenting the generation of *C. parapsilosis* lipase knockout mutants found that these have a decreased ability to form biofilms. *Candida parapsilosis* mutants produced significantly less biofilm than a wild type strain (Gácser *et al.,* 2007), and the *BCR* (biofilm and cell wall regulator) gene was necessary for proper biofilm formation (Ding *et al.,* 2007). Notably, the biofilm deficient *C. parapsilosis* lipase mutants were less virulent in tissue culture infection models and in mice (Gácser *et al.,* 2007). Lattif *et al.* (2010) demonstrated that, similarly to *C. parapsilosis*, the two newly identified *Candida* species (*C. orthopsilosis* and *C. metapsilosis*) were able to form biofilms.

Candida tropicalis clinical isolates have been classified as being strong biofilm formers (Negri *et al.,* 2010 a, c). Furthermore, Al-Fattani *et al.* (2006) showed that matrix material extracted from biofilms of *C. tropicalis* and *C. albicans* contained carbohydrates, proteins, hexosamine, phosphorus and uronic acid. However, the major component in *C. tropicalis* biofilm matrices was hexosamine (27%). The same authors also reported that these biofilms were partially detached after treatment with lipase type VII and chitinase, which contrasted with biofilms of *C. albicans* that were detached after treatment with proteinase K, chitinase, DNase I or B-N-aceytyglucosamidase.

The studies described highlight the diversity found in terms of biofilm forming ability, structure, and matrix composition concerning *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. It must be emphasized that it is highly

important to continue these studies for elucidating such inherent differences and the possibility to identify and especially combat strains adapted to infection at particular body sites.

Secreted enzymes by non-*Candida albicans Candida* species

The secretion of enzymes (proteases and phospholipases), as well as haemolytic activity (Figure I.4), are recognized as important factors in tissue invasion by *Candida* and the ability to secrete these enzymes has been associated with high pathogenicity.

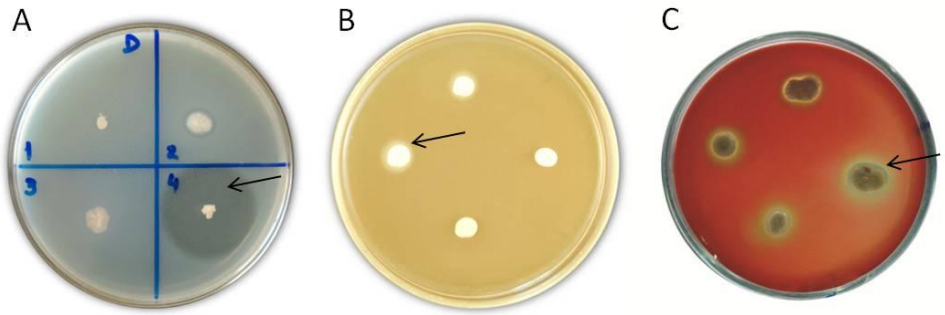

Figure I.4 Photographs showing secreted enzymes by *Candida* species with an opaque halo of degradation around the colonies grown in a specific agar plate. **A)** Proteinase activity on agar medium supplemented with BSA; **B)** Phospholipase activity on agar medium supplemented with egg; **C)** Heamolytic activity on agar medium supplemented with blood sheep and glucose .

The function of hydrolytic enzymes is associated with the destruction of host tissues and recently, some authors described that *Candida* has been able to degrade hemoglobin using haemolysins in order to obtain iron, (Luo, *et al.,* 2001, Naglik *et al.,* 2004; Negri *et al.,* 2010). These enzymes are most frequently described for *C. albicans*, and little is known

about their production and secretion by NCAC species and subsequent role in pathogenecity.

Secreted aspartyl proteinases (Saps)

The secretion of aspartyl proteinases (Sap1p to Sap10p) is an important virulence determinant of *C. albicans* (Naglik *et al.,* 1999; Hube *et al.,* 2001; Monod *et al.,* 2002). Saps facilitate invasion and colonization of host tissues by disruption of the host mucosal membranes (Rüchel *et al.,* 1992) and by degrading important immunological and structural defence proteins (Pichová *et al.,* 2001).

Concerning *C. glabrata*, only a single study has shown that this species is capable of proteinase production, but the type of proteinase was not specified (Chakrabarti *et al.,* 1991).

In addition, when compared to *C. albicans*, *C. parapsilosis* has a low Sap activity (Kobayashi *et al.,* 2004). Only three *SAP* genes have been identified in *C. parapsilosis* (*SAPP1-3*), two of which remain largely uncharacterized (Merkerova *et al.,* 2006). The Sapp1p isoenzyme has however been biochemically characterized (Fusek *et al.,* 1994; Pichová *et al.,* 2001; Dostal *et al.,* 2005) and *SAPP2*, produces a functional proteinase which constitutes about 20% of the Saps isolated from a culture supernatant (Fusek *et al.,* 1993). No studies have analyzed or characterized *SAPP2* or Sapp3. However, there is a trend relating Sap production and site isolation, since both vaginal and skin isolates of *C. parapsilosis* exhibit higher *in vitro* Sap activity than blood isolates (Cassone *et al.,* 1995; Dagdeviren *et al.,* 2005).

As with *C. albicans*, *in vitro* studies reveal that *C. tropicalis* secretes high levels of Saps in a medium containing bovine serum albumin (BSA) as sole source of nitrogen (Figure I.4 A). Furthermore, it is known that *C.*

tropicalis possesses at least four genes encoding Saps, and these are designated *SAPT1* to *SAPT4* (Togni *et al.,* 1991; Zaugg *et al.,* 2002). To date, Sapt1p is the only enzyme that has been purified from culture supernatant, biochemically characterized and crystallized (Togni *et al.,* 1991; Symersky *et al.,* 1997). The presence of aspartic proteinases secreted by *C. tropicalis* has also been reported on the surface of fungal elements penetrating tissues during disseminated infection and evading macrophages after phagocytosis of yeast cells (Borg *et al.,* 1992, Rüchel *et al.,* 1992).

Recently, Naglik *et al*. (2008) and Lerman *et al*. (2008) have demonstrated that, *SAP* gene expression on oral environment was not correlated with invasion and tissue damage.

Phospholipases and lipases

In addition to Saps, enzymes categorized as phospholipases (PLs) are often considered to be factors in *Candida* pathogenicity. PLs are enzymes that hydrolyze phospholipids into fatty acids. According to the different and specific ester bonds cleaved, these enzymes have been classified into PLs A, B, C and D (Mukherjee *et al.,* 2001). The production of all classes of PLs has been described for *Candida* species and their production could contribute to host cell membrane damage which could promote cell damage or expose receptors to facilitate adherence (Ghannoum *et al.,* 2000; Kantarcioglu *et al.,* 2002). The most widely used diagnostic method for phospholipase determination is based on the growth on egg yolk agar media (Figure I.4 B). Several studies indicate that NCAC species are able to produce extracellular phospholipases (Furlaneto-Maia, *et al.,* 2007; Cafarchia, *et al.,* 2008; Galan-Ladero, *et al.,* 2010), or relative significantly smaller amounts of phospholipase compared to *C. albicans* (Ghannoum *et al.,* 2000). There have been contradictory findings, with some investigators reporting phospholipase activity in 51% of strains assayed (Ghannoum *et*

al., 2000) and others finding no PLs activity at all (Kantarcioglu *et al.,* 2002). According to recent studies, whilst *C. tropicalis* appears to have a reduced ability to produce extracellular phospholipases, this production is strongly strain dependent (Cafarchia, *et al.,* 2008; Furlaneto-Maia, *et al.,* 2007; Galan-Ladero, *et al.,* 2010; Negri *et al.,* 2010). Furthermore, contrary to the few studies on *C. tropicalis* and *C. parapsilosis*, no studies have been reported concerning *C. glabrata* PLs production (Kumar *et al.,* 2009).

Lipases are involved in both the hydrolysis and synthesis of triacylglycerols and are characterised by having stability at high temperatures and in organic solvents, and being resistant to proteolysis (Brockerhoff *et al.,* 1974). In *C. albicans*, 10 lipase genes have been identified (Hube *et al.,* 2000). In *C. parapsilosis*, two lipase genes, *CpLIP1* and *CpLIP2* are reported with CpLIP2 known to encode for an active protein (Neugnot *et al.,* 2002; Brunel *et al.,* 2004). Recently, Gácser *et al.* (2007) demonstrated that a lipase inhibitor significantly reduced tissue damage during *C. parapsilosis* infection of reconstituted human tissues and that *CpLIP1-CpLIP2* mutants formed thinner and less complex biofilms. They also had reduced growth in lipid rich media were more efficiently ingested and killed by macrophage like cells, and were less virulent in infections of reconstituted human oral epithelium. Recent genomic DNA sequencing projects suggest that two additional *CpLIP* genes may exist in *C. parapsilosis* (Trofa *et al.,* 2008). Sequences similar to *C. albicans* (LIP1-10) were also detected in other pathogenic *Candida* species such as *C. tropicalis* but not in *C. glabrata* (Filler *et al.,* 1991). However, no relevant studies have been performed concerning the role of these genes on virulence of this species.

Haemolytic activity

Pathogenic microorganisms can grow in the host by using haemin or hemoglobin as a source of iron. The haemolysins are used by *Candida* species for degrading hemoglobin and extracting the elemental iron from host cells (Figure I.4 C). Thus, haemolysins are key virulence factors enabling pathogen survival and persistence in the host (Manns *et al.* 1994; Watanabe *et al,.* 1999; Luo *et al*., 2001). Furthermore, it is known that *C. albicans* possesses the ability to utilize iron to produce a factor which can release hemoglobin by lysing erythrocytes (Manns *et al.,* 1994; Watanabe *et al.,* 1999). Production of this haemolytic factor may be regulated by the presence of glucose in the growth medium. Furthermore, despite the activity being species and strain dependent, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* are also capable of *in vitro* production of one or more types of haemolysins (in an absent, partial or total manner) (Luo *et al*., 2001). Although others authors observed that *C. albicans, C. glabrata*, *C. tropicalis* and *C. parapsilosis* are cable to product total haemolysins after 48 hours of incubation (Bonassoli *et al.,* 2005; Furlaneto-Maia, *et al.,* 2007; Kumar, *et al.,* 2009; Negri *et al.,* 2010). Although haemolysins are known to be putative virulence factors contributing to pathogenicity in *Candida* species, the genetic expression of haemolytic activity of *Candida* is ill understood at present, but a study conducted for Luo *et al.* (2004) showed that *HLP* (haemolysin like protein) gene can be related with haemolytic activity of *C. glabrata*.

Concluding remarks

Changes in the host are generally required for opportunistic pathogens to switch from harmless commensal microorganisms to potentially life-threatening human pathogens. Opportunistic pathogens utilize several genes which play an important role in adhesion, biofilm formation, and enzymes secretion and are consequently involved in virulence. Given these findings and the increase incidence of candidosis caused by NCAC species, especially, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* and the unacceptable high morbidity and mortality, it is essential to increase our knowledge on the virulence factors associated with NCAC species. Studies in this area will also contribute towards the identification of new targets for future therapeutics against these recently emerged pathogens.

References

Ai-Said D, Anaissie E, Uzun O, *et al.,* (1997). The epidemiology of hematogenous candidiases caused by different Candida species. *Clin Infect Dis* **24**: 1122-1128.

Al-Fattani MA, Douglas LJ (2006). Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* **55**: 999-1008.

Almirante B, Rodriguez D, Cuenca-Estella M, *et al.,* (2006). Epidemiology, risk factors and prognosis of Candida parapsilosis bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003. *J Clin Microb* **44**: 1681-1685.

Àlvarez-Lerman F, Nolla-Sallas J, Léon C, *et al.,* (2003). Candiduria in critically ill patients admitted to intensive care units. *Intensive Care Med* **29**: 1069-1076.

Anil S, Ellepola AN, Samaranayake LP (2001). The impact of chlorhexidine gluconate on the relative cell surface hydrophobicity of oral *Candida albicans*. *Oral Dis* 7: 119-122.

Baillie GS, Douglas LJ (2000). *Matrix polymers of Candida biofilms and their possible role in biofilm resistance to antifungal agents. J Antimicrob Chemother* **46**: 397-403.

Bassetti M, Righi E, Costa A, *et al.,* (2006). Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infect Dis* **6**: 21.

Benjamin DK, Ross K, McKinney E, *et al.,* (2000). When to suspect fungal infection in neonates: a clinical comparison of Candida albicans and Candida parapsilosis fungeamia with coagulase-negative staphylococcal bacteremia. *Pediatrics* **106**: 712-718.

Binelli CA, Moretti ML, Assis RS, *et al.,* (2006). Investigation of the possible association between nosocomial candiduria an*d candidaemia. Clin Microbiol Infect* **12**: 538-543.

Bonassoli LA, Bertoli M, Svidzinski TIE (2005). High frequency of *Candida parapsilosis* on the hands of healthy hosts. *J Hosp Infect* **59:** 159-162

Borg M, Rüchel R (1992). Demonstration of fungal proteinase during phagocytosis of *Candida albicans* and *Candida tropicalis*. *J Med Vet Mycol* **28**:3-14.

Brito LR, Guimareaes T, Nucci M, *et al.,* (2006). Clinical and microbiological aspects of candidemia due to *Candida parapsilosis* in Brazilian tertiary care hospitals. *Med Mycol* **44**: 261-266.

Brockerhoff H (1974). Model of interaction of polar lipids, cholesterol, and proteins in biological membranes. *Lipids* **9**: 645-650.

Brunel L, Neugnot L, Landucci L, *et al.,* (2004). High-level expression of *Candida parapsilosis* lipase/acyltransferase in *Pichia pastoris*. *J Biochemical* **111**:41-50.

Butler G, Rasmussen MD, Lin MF, *et al.,* (2009). Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459**: 657-662.

Cafarchia, C, Romito D, Coccioli C, *et al.,* (2008). Phospholipase activity of yeasts from wild birds and possible implications for human disease. *Med Mycol* **46**: 429-434.

Cai S, Simionato MR, Mayer MP, *et al.,* (1994). Effects of subinhibitory concentrations of chemical agents on hydrophobicity and in vitro adherence of *Streptococcus mutants* and *Streptococcus sanguis*. *Caries Res* **28**: 335-341.

Calderone, RA (2002). Introduction and historical perspectives. In: Calderone R.A. (Eds.) Candida and candidiasis, ASM Press, Washington D.C., pp 15-25.

Camacho DP, Gasparetto A, Svidzinski TIE (2007). The effect of chlorhexidine and gentian violet on the adherence of *Candida* spp. to urinary catheters. *Mycopathologia* **163**:261-266.

Cassone A, De F Benardis, Pontieri E, *et al.,* (1995). Biotype diversity of *Candida parapsilosis* and its relationship to the clinical source and experimental pathogenicity. *J Infect Dis* **171**: 967-975.

Chaffin WL (2008). *Candida albicans* cell wall proteins. *Microbiol Mol Biol Rev* **72**: 495-544.

Chakrabarti A, Nayak N, Talwar P (1991). *In vitro* proteinase production by *Candida* species. *Mycophatologia* 114:163-168.

Chakrabarti A, Chatterjee SS, Rao KLN, *et al.,* (2009). Recent experience with fungaemia: change in species distribution and azole resistance. *Scand J Infect Dis* **41**:275-284.

Chandra J, Kuhn DM, Mukherjee PK, *et al.,* (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture and drug resistance. *J Bacteriol* **183**: 5385-5394.

Chen CM, Ho MW, Yu WL, *et al.,* (2006). *Candida parapsilosis* peritonitis has more complications than other *Candida* peritoneal dialysis patients. *Renal Fail* **28**: 241-246.

Colombo AL, Guimarães T, Silva IR, *et al.,* (2007). Prospective observational study of candidemia in Sao Paulo, Brazil: incidence rate, epidemiology, and predictors of mortality. *Infect Control Hosp Epidemiol* **28**: 570-576.

Colombo AL, Perfect J, DiNubile M, *et al.,* (2003). Global distribution and outcomes for *Candida* species causing invasive candidiasis: results from an international randomized double-blind study of caspofungin versus amphotericin B for the treatment of invasive candidiasis. *Eur J Microbiol Infect Dis* **22**: 470-474

Cormack BP, Ghori N, Flakow S (1999). An adhesion of the yeast *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285**: 578-582.

Costa-de-Oliveira S, Pina Vaz C, Mendonca D, *et al.,* (2008). A first Portuguese epidemiological survey of fungaemia in a university hospital. *Eur J Clin Microbiol Infect Dis* **27**: 365-374.

Crump A, Collignon PJ (2000). Intravascular catheter-associated infections. *Eur J Clin Microbiol Infect Dis* **19**, 1-8.

Dagdeviren M, Cerikcioglu N, Karavus M (2005). Acid proteinase, phospholipase and adherence properties of *Candida parapsilosis* strains isolated from clinical specimens of hospitased patients. *Mycoses* **48**: 321-326.

De las Peñas A, Pan PJ, Castano I, *et al.,* (2003). Virulence-related surface glycoproteins in the yeast pathogen *Candida glabrata* are encoded in subtelomeric clusters and subject to RAP1-and SIR dependent transcriptional silencing. *Genes Dev* **17**: 2245-2258.

Din C, Butler G (2007). Development of a gene knockout system in *Candida parapsilosis* reveals a conserved role for *BCR1* in biofilm formation. *Eukaryot* **6**: 1310-1319.

Domergue R, Castano I, De Las Peñas A, *et al.,* (2005). Nicotine acid limitation regulates silencing of *Candida glabrata* adhesins during UTI. *Science* **308**: 578-582.

Donlan RM, Costerton JW (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**: 167-193.

Dorko E, Pilipcinec E, Tkáciková L (2002). Candidal urinary tract infections caused by non-albicans Candida species. *Folia Microbiol (Praha*) **47**:182-184.

Dostal J, Dlouha H, Malon P, *et al.,* (2005). The precursor of secreted aspartic proteinase Sapp1p from *Candida parapsilosis* can be activated both autocatalytically and by a membrane-bound processing proteinase. *Biol Chem* 386: 791-799.

Eggimann P, Garbino J, Pittet D (2003). *Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. Lancet Infect Dis* **3**: 685-702.

Fidel PL, Vazquez J, Sobel JD (1999). *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison *C. albicans*. *Clin Microbiol Rev* **12:** 80-96.

Filler SG, Ibe BO, Luckett PM, *et al.,* (1991). *Candida albicans* stimulates endothelial cell eicosanoid production. *J Infect Dis* **164**: 928-935.

Furlaneto-Maia L, Specian AF, Bizerra FC *et al.* (2007). *In Vitro* evaluation of putative virulence attributes of oral Isolates of *Candida spp.* Obtained from Elderly Healthy Individuals. *Mycophatologia* **166**: 209-217.

Fusek M, Smith EA, Monod M, *et al.,* (1994). Extracellular aspartic proteinases from *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* yeasts differ substantially in their specificities. *Biochemistry* 33: 9791-9799.

Fusek M, Smith EA, Monod M, *et al.,* (1993). *Candida parapsilosis* expresses and secrets two aspartic proteinases. *FEBS Lett* 327: 108-112.

Gácser A, Schaffer W, Nosanchuk JS, *et al.,* (2007a). Virulence of *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* in a reconstituted human tissue models. *Fungal Genetic Biol* **44**: 1336-1341.

Gácser A, Trofa D, Schafer W, *et al.,* (2007b). Targeted gene deletion in *C. parapsilosis* demonstrates the role of secreted lipase in virulence. *J Clin Invest* **117**: 3049-3058.

Galan-Ladero MA, Blanco MT, Sacristán B, *et al.,* (2010). Enzymatic activities of Candida tropicalis isolated from hospitalized patients. *Med Mycol* **48**: 207-210.

Ghannoum MA (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* **13**: 122-143.

Gonzalez GM, Eliondo M, Ayala J (2008). Trends in species distribution and susceptibility of bloodstream isolates of *Candida* collected in Monterrey, Mexico, to seven antifungal agents: results of a 3-years (2004 to 2007) surveillance study. *J Clin Microbiol* **46**: 2902-2905.

Gonzalez GH, Gonzalez EM, Zambrano O, *et al.,* (2007). Oral Candidiasis in children and adolescents with cancer. Identification of Candida spp. *Med Oral Patol Oral Cir Bucal* **12:** 419-23

Hajjeh RA, Sofair AN, Harrison LH, *et al.,* (2004). Incidence of bloodstream infections due to *Candida* species and *in vitro* susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J Clin Microbiol* **42**:1519–1527.

Hargety JA, Ortiz J, Reich D, Manzarbeitia C (2003). Fungal infections in solid organ transplant patients. *Surg Infect (Larchmt)* **4**: 263-271

Harris AD, Castro J, Sheppard DC, *et al.,* (1999). Risk factors for nosocomial candiduria due to *Candida glabrata* and *Candida albicans*. *Clin Infect Dis* **29**: 926-928.

Hasan F, Xess I, Wang X, *et al.,* (2009). Biofilm formation in clinical isolates and its association with virulence. *Microbes Infect* **11**: 753-761.

Hazen KC, Plotkin BJ, Klima DM (1986). Influence of growth conditions on cell surface hydrophobicity of *Candida albicans* and *Candida glabrata*. *Infect Immun* **54**: 269-271.

Henriques M, Gasparetto K, Azeredo J, *et al.,* (2002). Experimental methodology to quantify *Candida albicans* cell surface hydrophobicity. *Biotechnol lett* **24**, 1111-1115.

Hube B, Naglik J (2001). *Candida albicans* proteinases: resolving the mystery of a gene family. *Microbiology* **147**: 1997-2005.

Hube B, Stehr F, Bossenz M, *et al.,* (2000). Secreted lipases of *Candida albicans*: cloning characterization and expression analysis of a new gene family with least ten members. *Arch Microbiol* **174**: 362-374.

Isenberg HD, Tucci V, Cintron F, *et al.,* (1989). Single source outbreak of Candida tropicalis complicating coronary bypass surgery. *J Clin Microbiol* **27**: 2426-2428.

Jain N, Kohli R, Cook GP, *et al.,* (2007). Biofilm formation by and antifungal susceptibility of *Candida* isolates from urine. *Appl Environ Microb* **73**, 1697-1703.

Joachim H, Polayes SH (1940). Subacute endocarditis and systemic mycosis (monilia). **JAMA** 205-208.

Kantarcioglu AS, Yucel A (2002). Phospholipase and protease activity in clinical *Candida* isolates with reference to the sources of strains. *Mycoses* **45**: 160-165.

Kauffman CA, Vasquez JA, Sobel JD, *et al.,* (2000). Prospective multicenter surveillance study of funguria in hospitalized patients. *Clin Infect Dis* 30: 14-18.

Kiehn TE, Edwards FF, Armstrong D (1980). The prevalence of yeasts in clinical specimens from cancer patients. *Amer J Clin Pathol* **73**: 518-52.

Kikutaní H, Makino S (1992). The murine autoimmune diabets model: NOD and related strains. *Adv Immunol* **51**: 285-322.

Klotz SA, Drutz D, Zajic JE (1985). Adherence and penetration of vascular endothelium by *Candida* yeast. *Infect Immun* **42**: 955-978.

Kobayashi CC, Fernandes OF, Miranda KC, *et al.,* (2004). Candiduria in hospital patients: a study prospective. *Mycophatologia* **158**: 49-52.

Kojic EM, Darouiche RO. (2004). *Candida* infections of medical devices. *Clin Microbiol Rev* **17**: 255–267.

Kontoyiannis DP, Vaziri IH, Hend A, *et al.,* (2001). Risk factors for *Candida tropicalis* fungemia in patients with cancer. *Clinical Infectious Diseases* **33**, 1676-1681.

Kossoff EH, Buescher ES, Karlowiez MG (1998). Candidemia in a neonatal intensive care unit: trends during fifteen years and clinical features of 111 cases. *Pediatr Infect Dis* **17**: 504-508.

Krcmery V (1999). *Candida (Toluropsis) glabrata*–an emerging pathogen in cancer patients. *Int J Antimicrob Agents* **11**: 1-6.

Krcmery V, Barnes AJ (2002). *Non-albicans Candida spp. causing fungaemia: pathogenicity and antifungal resistance. J Hosp Infect* **50**: 243-260.

Kuhn DM, Chandra J, Mukherjee PK, *et al.,* (2002). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun* **70**: 878-888.

Kumamoto CA (2002). Candida biofilms. *Curr Opin Microbiol* **5**: 608-611.

Kumar, V.G., R. Latha, K. Vedhagiri *et al.*, (2009). Phospholipase C, proteinase and hemolytic activities of *Candida* spp. isolated from pulmonary tuberculosis patients. *J Med Mycol* **19**:3-10

Larone DH (2002). Medically important fungi; a guide to identification; 4th ed. Washington: ASM Press, 409.

Lass-Flörl (2009). The Changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* **52**:197-205.

Lattif AA, Mukherjee PK, Chandra J, *et al.,* (2009). Characterization of biofilms formed by *Candida parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*. *Inter J Med Microbiol* Doi:10.1016/j.ijmm2009.09.001.

Lerman U, Morschhauser J (2008). Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*. *Microbiol* **154**: 3281-3295.

Liguori G, Di Onofrio V, Lucariello A, *et al.,* (2009). Oral candidosis: a comparasion between conventional methods and multiplex polymerase chain reaction for species identification. *Oral Microbiol Immun* **24**: 76-78.

Luo G, Samaranayake LP, Chenng BP, *et al.,* (2004). Reverse transcriptase polymerase chain reaction (RT-PCR) detection of *HLP* gene expression in *Candida glabrata* and its possible role in vitro haemolysin production. *APMIS* **112**: 283-290.

Luo G, Samaranayake LP (2002) *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. *APMIS* **119**: 601-610.

Luo G, Samaranayake LP, Yau JYY (2001). *Candida Species* exhibit differential in vitro hemolytic activities. *J Clin Microbiol* **39**: 2971-2974.

Luque AG, Biasoli MS, Tosello ME, *et al.,* (2008). Oral yeast carriage in HIV-infected and non-infected populations in Rosario, Argentina. *Mycoses 52*: 53-59.

Manns JM, Mosser DM, Buckley HR (1994). Production of a hemolytic factor by *Candida albicans. Infect Immun* **62**: 5154-5156.

Manzano-Goyosso P, Hernández–Hernández F, *et al.,* (2008). Candiduria in type 2 diabets mellitus patients and its clinical significance. *Candida* spp antifungal susceptibility. *Rev Med Inst Mex Seguro Soc* **46**: 603-610.

Martin MV (1979). Germ-tube formation by oral strains of *Candida tropicalis.* J *Med Microbiol* **12**(2): 187-194.

Martins M, Henriques M, Fernandes R, *et al.,* (2010 a). Oral carriage of patients attending a dental clinic in Barga, Portugal. *Rev Iberoam Micol* (In Press)

Martins M, Henriques M, Azeredo J, *et al.,* (2010b). *Candida* species extracellular alcohols: production and effect in sessile cells. *J Bas Microbiol*, doi:10.1002/jobm.200900442.

Merkerova M, Dostal J, Hradilek M, *et al.,* (2006). Cloning and characterization of Sapp2p, the second aspartic proteinase isoenzyme from Candida parapsilosis. *FEMS Yeast Res* **6:** 1018-1026.

Messer SA, Jones RN, Fritsche TR (2006). International surveillance of Candida spp. and Aspergillus spp: report from the SENTRY Antimicrobial Surveillance Program. *J Clin Microbiol* **44**: 1782-1787.

Miranda LN, van der Heijden, Costa SF, *et al.,* (2009). *Candida* colonisation as a source for Candidemia. *J Hosp Infect* **77**: 9-16.

Monod M, Borg-von-Zepelin (2002). Secreted aspartic proteases as virulence factors of Candida species. *Biol Chem* **383**: 1087-1093.

Mukherjee PK, Chandra J (2004). *Candida* biofilm resistance. *Drug Resist update* **7**: 301-309.

Mukherjee PK, Seshan KR, Leidich SD, *et al.,* (2001). Reintroduction of the PLB1 gene into *Candida albicans* restores virulence in vivo. *Microbiol* **147**:2585-97.

Naglik JR, Newport G, White TC, *et al.,* (1999). *In vivo* analysis of secreted aspartyl proteinase expression in human oral candidiases. *Infect Immun* **67**: 2482-2490.

Naglik JR, Albrecht A, Bader O, *et al.,* (2004). Candida albicans proteinases and host/pathogen interactions. *Cell Microbiol* **6**: 915-926.

Naglik JR, Moyes D, Makwana J, *et al*., (2008). Quantitative expression of *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiol* **154**: 3266-3280.

Negri M, Martins M, Henriques M, *et al.,* (2010a). Examination of potential virulence factors of *Candida tropicalis* clinical isolates from hospitalized patients. *Mycophatologia* Doi: 10.1007/s11046-009-9246-0.

Negri M, Gonçalves V, Silva S, *et al.,* (2010b). Crystal violet expedite method for quantification adhesion to epithelial cells. *Bri J Microbiol*. (In press).

Negri M, S Silva, Henriques M, *et al.,* (2010c). Simple *model for Candida tropicalis biofil*m *in vitro* studies using flow conditions, urinary catheters and artificial urine. (Submitted).

Neugnot V, Moulin G, Dubreucq E, *et al.,* (2002). The lipase/acyltransferase from *Candida parapsilosis*: molecular cloning and characterization of purified recombinant enzymes. *Eur J Biochem* **269**: 1734-1745.

Nucci M, Colombo AL (2007). Candidemia due to *Candida tropicalis*: clinical, epidemiologic and microbiologic characteristics of 188 episodes occurring in tertiary care hospitals. *Diagn Microbiol Infect Dis* **58**: 77-82.

Odds FC (1988). *Candida* and candidiasis, 2nd edn London: Bailliere Tindall.

Panogoda GJ, Ellepola NB, Samaranayake LP (2001). Adhesion of *C. parapsilosis* to epithelial and acrylic surfaces correlates with the cell surface hydrophobicity. *Mycoses* **44**: 29-35.

Passos XS, Sales WS, Maciel PJ, *et al.,* (2005). *Candida* colonization in intensive care unit patients' urine. *Memórias do Instituto Oswaldo Cruz* **100**: 925-928.

Pfaller MA, Diekema DJ (2007). Epidemiology of Invasive Candidiasis*:* a Persistent Public Health Problem. *Clin Microbiol Rev* **20**: 133-163.

Pfaller MA, Jones RN, Messer SA, *et al.,* (1998). National surveillance of nosocomial bloodstream infection due to *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagnostic Microbiology and Infectious Disease* **31**: 327-332.

Pichová I, Pavlicková L, Dostal J*, et al.,* (2001). Secreted aspartic proteases of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida lusitaniae*. Inhibition with peptidomimetic inhibitors. *Eur J Biochem* **268**: 2669-2677.

Ramage G, Martínez JP, López-Ribot JL (2006). *Candida* biofilms on implanted biomaterials: a clinically significant problem*. FEMS Yeast Res* **6**, 979-986.

Redding SWW, Kirkpatrick R, Coco BJ, *et al.,* (2002). *Candida glabrata* oropharyngeal candidiasis in patients receiving radiation treatment for head and neck cancer. *J Clin Microbiol* **40**:1879–1881.

Rho J, Shin JH, Song JW, *et al.,* (2004). Molecular investigation of two consecutive nosocomial clusters of *Candida tropicalis* candiduria using pulsed-field gel electrophoresis. *J Microbiol* **42**: 80-86.

Richards MA, Edwards JR, Culver DH, *et al.,* (1999). Nosocomial infections in medical intensive care units in the United States. Notational Nosocomial Infections Surveillance System. *Crit Care med* **27**: 887-892

Ruan SY, Chen SY, Husek PR (2008). Persistent *Candida parapsilosis* funguria associated with indwelling urinary tract stent for more than 7 years. *J Med Microbiol* **57**: 1585-1587.

Rüchel R, Bernardis F, Ray TL, *et al.,* (1992). *Candida* acid proteinases. *J Med Vet Mycol* **30**:123-132.

Rüping MJ, Vehreschild JJ, Cornely OA (2008). Patients at high risk of invasive fungal infections: when and how to treat. *Drugs* **68**: 1941-62.

Samaranayake LP, Fidel PL, Naglik JR, *et al.,* (2002). Fungal infections associated with HIV infection. *Oral Dis* **8**: 151-160.

Saimain L, Ludington E, Dawson JD, *et al.,* (2001). Risk factors for *Candida* colonization of neonatal intensive care unit patients. *Pediatric Infect Dis* **20:** 1119-1124.

Schaller M, Zakikhany K, Naglik JR, *et al.,* (2006). Models of oral and vaginal candidiasis based on *in vitro* reconstituted human epithelia. *Nat Prot* **1**: 2767-2773.

Shin JH, Kee SJ, Shin MG, *et al.,* (2002). Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from others sources. *J Clin Microbiol* **40**: 1244-1248.

Silva AP, Miranda IM, Lisboa C, *et al.,* (2009). Prevalence, distribution, and antifungal susceptibility profiles of *Candida parapsilosis, C. orthopsilosis*, and *C. metapsilosis* in a tertiary care hospital. *J Clin Microbiol* **47**: 2392-2397.

Sobel JD, Kauffman CA, McKinsey D, *et al.,* (2000). Candiduria: a randomized, double-blind study of treatment with fluconazole and placebo. *Clin Infect Dis* **30**:19-24.

Solomon SL, Khabbaz RF, Parker RH, *et al.,* (1984). An outbreak of *Candida parapsilosis* bloodstream infections in patients receiving parenteral nutrition. *J Infect Dis* **149:** 98-102.

Sousa C, Henriques M, Teixeira P, *et al.,* (2009). Influence of surface properties on the adhesion of *Staphylococcus epidermidis* to acrylic and silicone. *Int J Biomather* doi:10.1155/2009/718017.

Symersky J, Monod M, Foundling SI (1997). High-resolution structure of the extracellular aspartic proteina*se* from *Candida tropicalis* yeast. *Biochemistry* **36**:12700-12710.

Togni G, Sanglard D, Falcetto R, *et al.,* (1991). Isolation and nucleotide sequence of the extracellular acid protease gene (ACP) from the yeast *Candida tropicalis*. *FEBS Lett* **28**: 181-185.

Tortorano AM, Kibbler C, Peman J, *et al.,* (2006). Candidemia in Europe: epidemiology and resistance. *Int J Antimicrob Agents* **27**: 259-366.

Trofa D, Gácser A, Nosanchink JD (2008). *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* **21**: 606-625.

Vazquez JA, Sanchez C, Dmuchowski C, *et al.,* (1993). Nosocomial acquisition of *Candida albicans*: an epidemiology study. *J Infect Dis* **168**: 195-201.

Voss A, Hollis RJ, Pfaller MA, *et al.,* (1994). Investigation of the sequence of colonization and candidemia in nonneutropenic patients. *J Clin Microbiol* **32:** 975-980.

Watanabe T, Takano M, Murakami M, *et al.*, (1999). Characterization of a haemolytic factor from *Candida albicans. Microbiol* **145**: 689-694.

Weem JJ (1992). *Candida parapsilosis*: epidemiology, pathogenicity, clinical manifestations, and antimicrobial susceptibility. *Clin Infect Dis* **14**: 756-766.

Yoshio O, Kouji G (2006). Antigenicity of *Candida tropicalis* strain cells cultured at 27 and 37deg. *FEMS Immunol Med Microbiol* **46**:438-443.

Zaugg C, Zepelin MB, Reichard U, *et al.,* (2001). Secreted aspartic proteinase family of *Candida tropicalis*. *Infect Immun* **69**: 405-412.

Chapter II

Biofilms of *non-Candida albicans Candida* species: quantification, structure and matrix composition

The work presented in this chapter was published:

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Abstract

Most cases of candidosis have been attributed to *C. albicans*, but recently, non-*Candida albicans Candida* (NCAC) species have been identified as common pathogens. The ability of *Candida* species to form biofilms has important clinical repercussions due to their increased resistance to antifungal therapy and the ability of yeast cells within the biofilms to withstand host immune defenses. Given this clinical importance of the biofilm growth form, the aim of this study was to characterize biofilms produced by three NCAC species, namely *C. parapsilosis, C. tropicalis* and *C. glabrata.* The biofilm forming ability of clinical isolates of *C. parapsilosis*, *C. tropicalis* and *C. glabrata* recovered from different sources, was evaluated by crystal violet staining. The structure and morphological characteristics of the biofilms were also assessed by scanning electron microscopy and the biofilm matrix composition analyzed for protein and carbohydrate content. All NCAC species were able to form biofilms although these were less extensive for *C. glabrata* compared with *C. parapsilosis* and *C. tropicalis*. It was evident that *C. parapsilosis* biofilm production was highly strain dependent, a feature not evident with *C. glabrata* and *C. tropicalis*. Scanning electron microscopy revealed structural differences for biofilms with respect to cell morphology and spatial arrangement. *Candida parapsilosis* biofilm matrices had large amounts of carbohydrate with less protein. Conversely, matrices extracted from *C. tropicalis* biofilms had low amounts of carbohydrate and protein. Interestingly, *C. glabrata* biofilm matrix was high in both protein and carbohydrate content. The present work demonstrates that biofilm forming ability, structure and matrix composition are highly species dependent with additional strain variability occurring with *C. parapsilosis*.

Keywords: Biofilm; non-*Candida albicans Candida* species

Introduction

Invasive fungal infections, such as candidosis, represent a public health problem of major importance (Fridkin *et al.,* 1996). *Candida* species normally exist as commensals but they are opportunistic pathogens, with the ability to cause a variety of superficial and systemic infections (Odds, 1998). In the past ten years, the number of infections caused by *Candida* species has progressively increased (Samaranayake *et al.,* 2002). This emergence is often associated with the increasing incidence of human immunodeficiency virus (HIV) infection (Fanello *et al.,* 2001), the rise in the elderly population base (Hargety *et al.,* 2004), a higher number of immunocompromised patients and the more widespread use of indwelling medical devices (Kumamoto, 2002; Kojic *et al.,* 2004). Although most candidosis have been attributed to Candida albicans, more recently, non-*Candida albicans Candida* (NCAC) species (*Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata*) have been identified as common pathogens. The prevalence of these species in human infection has been changing in recent years. In the 1980s, according to Kiehn *et al.* (1980), *C. albicans* constituted 68% of *Candida* isolates from sites other than blood, in cancer patients, while *C. tropicalis*, *C. parapsilosis* and *C. glabrata* accounted only for 12.3, 10.3 and 3.0% of isolates, respectively. Moreover, recently, 60% of the fungemia cases reported by Bassetti *et al.* (2006) were due to NCAC species. *Candida tropicalis* has emerged as the second or third most common agent of candidemia, mainly in oncology patients (Weinberger *et al.,* 2005; Nucci *et al.,* 2007). Moreover, the increased incidence of *C. tropicalis* as a causative agent of nosocomial urinary tract infections has been reported (Rho *et al.,* 2004). *Candida parapsilosis* is generally regarded as one of the less virulent yeast species, although it is now a frequent cause of candidemia. Nosocomial outbreaks of *C.*

parapsilosis have also been described and have been attributed to transfer of yeast from the hands of healthcare workers (Bonassoli *et al.,* 2005). *Candida glabrata* has recently emerged as an important nosocomial pathogen, yet little is known about its epidemiology (Hajjeh *et al.,* 2004). *Candida glabrata* is of particular importance because of its innately high resistance to certain antifungal agents, specifically the azoles (Tsai *et al.,* 1997).

One of the major contributions to *Candida* virulence is its versatility in adapting to a variety of different habitats and the formation of surface-attached microbial communities known as biofilms (Costerton *et al.,* 1995). Biofilm cells are organized into structured communities embedded within a matrix of extracellular material that is produced by the biofilm cells (Donlan *et al.,* 2002). Generally, the biofilm matrix composition includes (in addition to water), carbohydrates, proteins, phosphorus, glucose and hexosamines. However, a large portion of the biofilm matrix still remains to be identified (Baillie *et al.,* 2000). The formation of *Candida* biofilms has important clinical repercussions because of their increased resistance to antifungal therapy and the protection afforded against host immune defenses (Donlan *et al.,* 2002; Mukherjee *et al.,* 2004).

Many previous studies have focused on *C. albicans* biofilms (Hawser *et al.,* 1995; Baillie *et al.,* 1999, 2000; Chandra *et al.,* 2001; Jin *et al.,* 2003; Ramage *et al.,* 2006) due to its well-recognized virulence, whereas only few studies of biofilms generated by all NCAC species have been reported (Hawser *et al.,* 1994; Shin *et al.,* 2002; Samaranayake *et al.,* 2005; Al-Fattani *et al.,* 2006; Jain *et al.,* 2007; Bizerra *et al.,* 2008). Thus, the aims of this work were firstly to assess biofilm formation ability of clinical isolates of *C. glabrata*, *C. tropicalis* and *C. parapsilosis* recovered from different body sites, and secondly, to characterize the biofilm structure and matrix composition in terms of protein and carbohydrate content.

Materials and methods

Organisms

A total of 18 clinical strains (Table II.1) of *C. tropicalis*, *C. glabrata* and *C. parapsilosis*, recovered from different body sites, were used in the course of this study. The majority of strains were recovered from vaginal and urinary tract samples were and were part of the collection at the Hospital of S. Marcos, Braga, Portugal. *Candida tropicalis* 12 and 75 (recovered from the vaginal tract), were obtained from the archive collection of the University of Maringá, Brazil. All oral isolates were stock isolates of the biofilm group of the Centre of Biological Engineering, and were originally isolated from Clinic of Dentistry, Congregados, Portugal. Three reference strains of *C. tropicalis*, *C. glabrata* and *C. parapsilosis* from the American Type Culture Collection (ATCC) were also examined. The identity of all isolates was confirmed using CHROMagar *Candida* (CHROMagar, Paris, France) and by PCR-based sequencing using specific primers (ITS1 and ITS4) against the 5.8s subunit gene reference. Genomic DNA was extracted following previously described procedures (Scherer *et al.,* 1987). The PCRs products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK).

Growth conditions

For each experiment, strains were subcultured on sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 48 h at 37°C. Cells were then inoculated in sabouraud dextrose broth (SDB) (Merck) and incubated for 18 h at 37°C under agitation at 120 rev/min. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min

at 4°C and washed twice with ultra-pure sterile water. Pellets were then suspended in SDB and the cellular density adjusted to $1x10^7$ cells ml^{-1} using a Neubauer counting chamber.

Biofilm biomass quantification

Standardized cell suspensions (200 µl of containing $1x10^7$ cells ml^{-1} in SDB) were placed into selected wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) and incubated at 37°C on a shaker at 120 rev/min. At 24 h, 100 µl of SDB medium was removed and an equal volume of fresh SDB added. The preparations were then incubated for a further 48 h. After this step, the medium was aspirated and non-adherent cells removed by washing the biofilms twice with sterile ultra-pure water.

Biofilm forming ability was assessed through quantification of total biomass by crystal violet (CV) staining. Thus, after washing, biofilms were fixed with 200 µl of methanol, which was removed after 15 min of contact. The microtiter plates were allowed to dry at room temperature, and 200 µl of CV (1% v/v) added to each well and incubated for 5 min. The wells were then gently washed with sterile, ultra-pure water and 200 µl of acetic acid (33% v/v) added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. Experiments were repeated as part of three to five independent assays.

Biofilm structure

To examine the structure of biofilms by scanning electron microscopy 2 ml of the standardized cell suspension ($1x10^7$ cells ml^{-1} in SDB) was introduced into 24-well polystyrene plates (Orange Scientific) and

incubated for 48 h at 37°C and 120 rev/min. After 24 h, 1 ml of SDB medium was removed and an equal volume of fresh SDB added. At 48 h, the medium was aspirated and non-adherent cells removed by washing the biofilms twice with sterile ultra-pure water. Samples were dehydrated with alcohol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. Samples were kept in a desiccator until the base of the wells was removed for analysis. Prior to observation, the base of the wells were mounted onto aluminium stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

Biofilm matrix composition

Extraction method

Biofilms for analysis of matrix material were formed in 6-well polystyrene microtiter plates (Orange Scientific). For this, inocula of 3 ml of yeast cell suspension ($1x10^7$ cells ml^{-1} in SDB) were added to each well and biofilms were formed as described previously. After 48 h, the biofilm matrix was extracted using a slight modification to a previously described protocol (Azeredo *et al.,* 2003). Briefly, biofilm samples were scraped from the 6-well plates, resuspended with ultra-pure water, sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W, and then the suspension was vortexed for 2 min. The suspension was centrifuged at 3000 *g* for 10 min at 4°C and the supernatant filtered through a 0.2 µm nitrocellulose filter and stored at -20°C before analysis. The pellets were dried at 60°C until a constant dry biofilm weight was determined. The experiments were performed in triplicate and in three independent assays.

Quantification assays

Protein and carbohydrate quantification

The protein content of the biofilm matrix was measured using the BCA Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, USA), using bovine serum albumin (BSA) as the standard.

Total carbohydrate content of the biofilm matrix was estimated according to the procedure of Dubois *et al.* 1956, using glucose as standard.

Statistical Analysis

Results were compared using One-Way analysis of variance (ANOVA) by applying Levene´s test of homogeneity of variance and the Tukey multiple-comparisons test, using SPSS software (SPSS [Statistical Package for the Social Sciences], Inc., Chicago, USA). All tests were performed with a confidence level of 95%.

Results

Biofilm forming ability by non-*Candida albicans Candida* species

Figure II.1 presents the results for biofilm quantification using CV staining. It was evident that all NCAC species formed biofilms, although differences occurred depending on species or strain as in the case of *C. parapsilosis*. Importantly, it was noticed that generally *C. glabrata* biofilms had less total biomass (average Abs=0.53±0.22) compared with *C. parapsilosis* (average Abs=1.14±0.43) and *C. tropicalis* (average Abs=1.31±0.08).

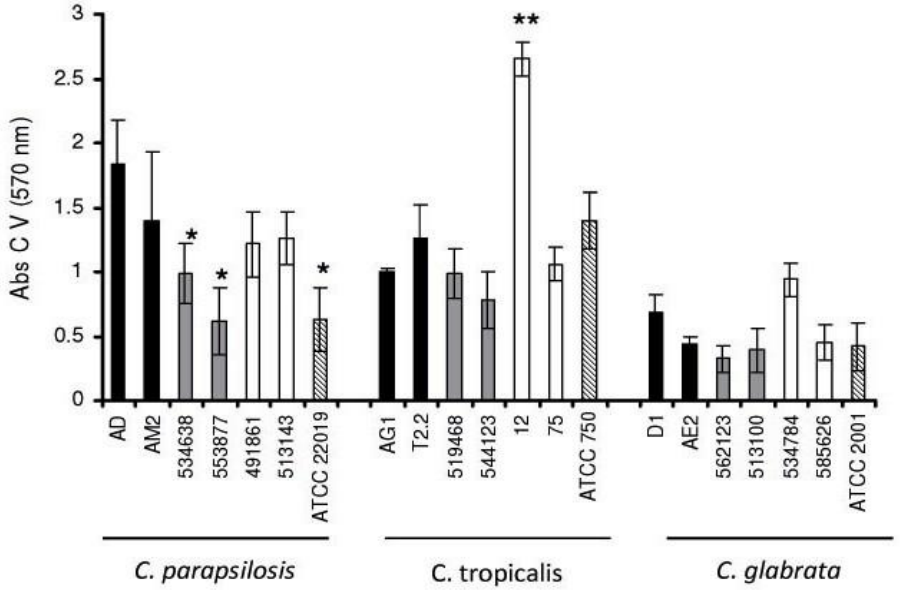

Figure II.1 Absorbance values of crystal violet solutions (Abs CV) obtained from 48 h biofilms of non-*Candida albicans Candida* species formed in SDB (λ=570 nm) from different origins (■ oral, ■ urinary and □ vaginal). Error bars represent standard deviation. *Strains that are significantly different (*P*<0.05) in each species.

Candida glabrata strains had similar biofilm forming ability with no significant strain differences (P>0.05). In contrast, *C. parapsilosis* strains were heterogeneous in terms of the level of biofilm formation. Biofilms formed by *C. parapsilosis* AD yielded the highest level of absorbance (Abs=1.84±0.34) which were statistically higher than for *C. parapsilosis* 534638 (P=0.004), 553877 (P<0.001) and ATCC 22019 (P<0.001). *Candida tropicalis* strains exhibited a more homogeneous behaviour with all strains being high level biofilm formers (Abs values ≥ 0.75). No statistical differences were found in the extent of biofilm formation for all the *C. tropicalis* strains (P>0.05), with the exception of strain 12, which had the highest biofilm forming ability (Abs=2.65±0.13) (P<0.001). This strain produced a two-fold greater level of biofilm than other strains of *C. tropicalis* and *C. parapsilosis*, and a five-fold increase over *C. glabrata* strains.

For each species, strains originating from the urinary tract generally yielded lower biofilm levels compared with those from other body sites. In the case of *C. parapsilosis,* oral isolates were the highest biofilm producers and for the other NCAC species studied, the highest biofilm producers were vaginal isolates (*C. glabrata* 534784 and *C. tropicalis* 12).

Structure of non-*Candida albicans Candida* species biofilms

SEM analysis was used to examine biofilm structure and to determine *Candida* morphological characteristics (Figure II.2).

Mature biofilms of *C. parapsilosis* and *C. tropicalis* strains consisted of a dense network of cells of a variety of morphologies. Biofilms of *C. parapsilosis* strains AD, 553877 and ATCC 22019 were composed of both yeasts and pseudohyphae, although biofilms formed by other strains of the same species were devoid of pseudohyphae (Figure II.2 A).

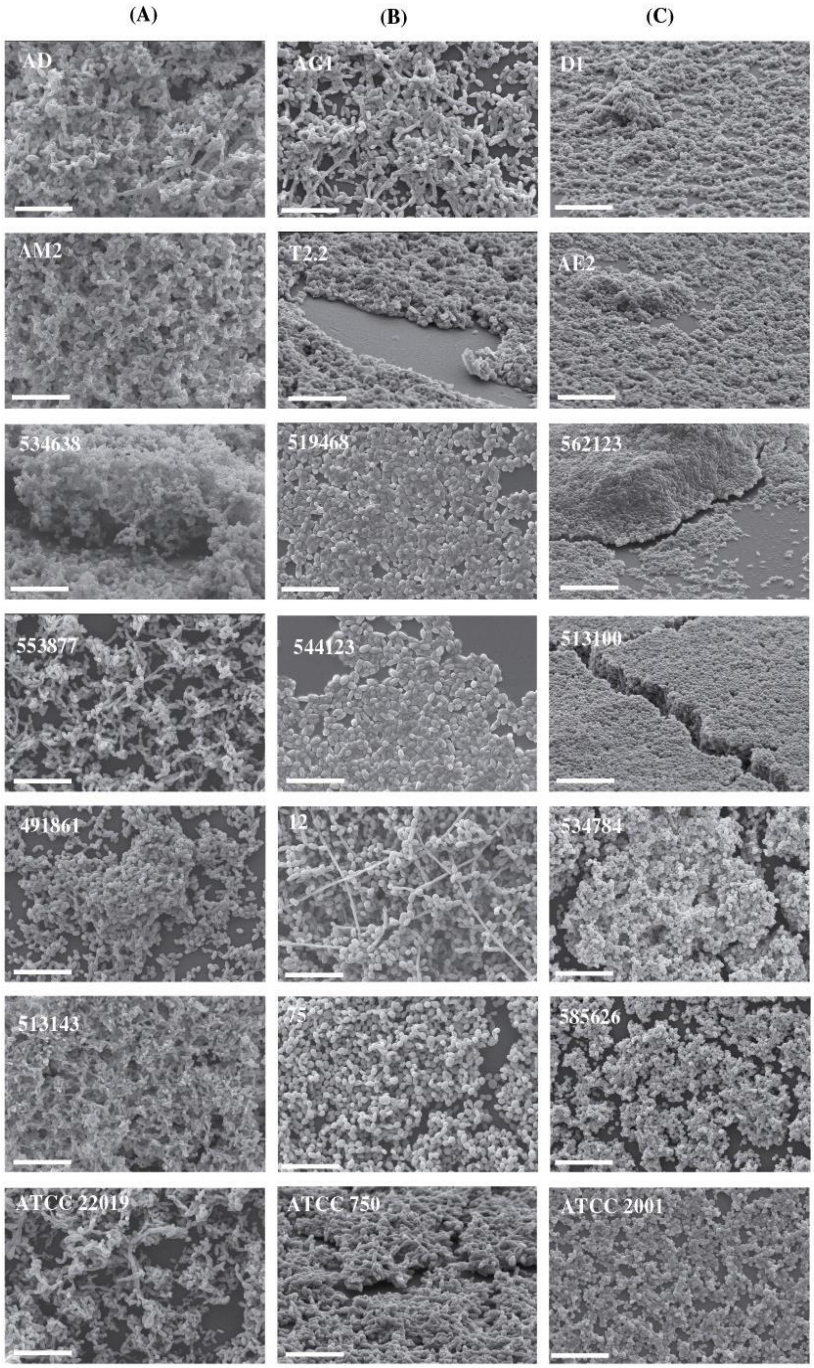

Figure II.2 Scanning electron microscopy of non-*Candida albicans Candida* species biofilms formed in SDB at 48 hours. **(A)** *C. parapsilosis*, **(B)** *C. tropicalis* and **(C)** *C. glabrata* clinical isolate strains. Arrows indicate the presence of hyphal morphologies. The bar in the images corresponds to 20 µm. Magnification ×1000.

Biofilms formed by *C. tropicalis* exhibited only yeasts morphology, with exception of strains AG1 and 12 which presented hyphal forms, with the latter appearing as especially long filaments (Figure II.2 B). All biofilms of *C. glabrata* strains were comprised only of yeasts (Figure II.2 C).

In the case of *C. parapsilosis* biofilm structure (Figure II.2 A), strains AD, AM2, 534638 and 513143 formed a multilayer and compact biofilm covering the entire surface. In contrast, biofilms of strains 553877, 491861 and ATCC 22019, consisted of non-contiguous cell aggregates. The structure of *C. tropicalis* (Figure II.2 B) biofilms was also strain dependent with some strains (AG1, T2.2, 12, 75 and ATCC 750) producing thick biofilms of co-aggregated cells and others (strain 519468 and 544123) yielding a more discontinuous monolayer of yeasts anchored to surface. *Candida glabrata* (Figure II.2 C) revealed either a multilayer biofilm structure intimately packed (strains 562123 and 513100) or constituted by clusters of cells (strains D1, AE2, 534784, 585626 and ATCC 2001).

The biofilm structures for isolates from the same clinical origin were similar for *C. glabrata* strains (Figure II.2 C). Biofilms formed by the urinary isolates (562123 and 513100) displayed a highly compact layer covering the entire surface in a "carpet" like appearance. Biofilms of oral isolates (D1 and AE2) revealed smaller clusters compared to those in biofilms of vaginal isolates (534784 and 585626). Biofilms of oral *C. parapsilosis* strains (Figure II.2 A) (AD and AM2) were very similar, presenting a multilayer structure. In case of *C. tropicalis* (Figure II.2 B) urinary isolates (519468 and 544123), the biofilms were also similar, presenting as discontinuous monolayer's.

Matrix biofilm composition

Table II.1 shows the yield of total protein and carbohydrates extracted from biofilms formed by the NCAC species studied.

The results showed that, generally *C. parapsilosis* biofilm matrices had high amounts of carbohydrate (average mg/g $_{\text{biofilm dry weight}}$ = 611.2±206) and relatively lower amounts of proteins (average mg/g $_{\text{biofilm dry weight}}$ = 50.9±21.4). Strain differences were evident in terms of both protein and carbohydrate contents, with C*. parapsilosis* AM2 having a statistically higher carbohydrate and protein content in its biofilm matrix as compared with the all other *C. parapsilosis* strains, except strain 491861 (the highest protein content) and strain AD (similar to AM2 in carbohydrate content). In contrast, compared with the other species, the biofilm matrices of *C. tropicalis* strains had lower concentrations of both protein (average mg/g $_{\text{biofilm dry weight}}$ = 43.4±12.7) and carbohydrates (average mg/g $_{\text{biofilm dry weight}}$ = 24.6±15.9). Despite statistical differences being evident between C*. tropicalis* strains, the relatively low protein and carbohydrate matrix composition was a consistent finding for *C. tropicalis*. Interestingly, biofilm matrices of *C. glabrata* had relatively higher quantities of both protein (average mg/g $_{\text{biofilm dry weight}}$ = 226.7±58.6) and carbohydrate (average mg/g $_{\text{biofilm dry weight}}$ = 431.3±179.6) compared with the other species. Indeed, protein levels were on average five times higher than those of *C. parapsilosis* and *C. tropicalis*.

No correlation was found concerning the amount of carbohydrate and protein extracted from biofilm of NCAC species and the respective source of each clinical isolate. However, the biofilm matrix of the vaginal clinical isolate, *C. tropicalis* 12, presented the highest amount of protein and carbohydrate of all clinical isolates of *C. tropicalis*. For *C. parapsilosis*, oral isolates (AD and AM2) had the highest quantity of carbohydrates and the urinary isolate, *C. parapsilosis* 534638, the lowest protein and carbohydrate content.

Table II.1 Origin, reference and biofilm matrix composition of non-*Candida albicans Candida* species. The values are means ± standard deviations

Species	Origin	Reference	Matrix component (mg/g of biofilm dry weight)	
			Protein	Carbohydrate
C. parapsilosis	Oral tract	AD	35.9±7.2	748.8±43.8*
		AM2	75.1±7.2*	926.8±144.9*
	Urinary tract	534638	20.2±4.5*	263.7±13.2*
		553877	46.8±16.6	592.6±93.4
	Vaginal	491861	80.6±16.6*	555.2±238.5
		513143	55.3±16.6	675.2±169.0
	Reference	ATCC 22019	42.2±10.3	516.4±219.1
C. tropicalis	Oral tract	AG1	46.3±3.5	22.2±5.8
		T2.2	28.2±3.3*	21.5±4.0
	Urinary tract	519468	34.2±9.3	15.7± 1.9
		544123	41.6±1.0	11.3±5.8
	Vaginal	12	54.0±2.1*	58.7±7.4*
		75	34.7±3.7	27.5±2.8
	Reference	ATCC 750	64.6±18.2*	15.5±2.8
C. glabrata	Oral tract	D1	325.2±31.4	572.8±111.2
		AE2	226.7±84.1	241.8±52.2
	Urinary tract	562123	181.7±28.7	409.5±112.4
		513100	226.5±59.3	233.7±88.5
	Vaginal	534784	136.4±38.5	398.3±130.8
		585626	246.9±47.5	742.6±285.2*
	Reference	ATCC 2001	243.6±30.7	420.3±39.2

* Significantly different (P<0.05) for each species.

Discussion

Biofilm forming ability may confer NCAC species an ecological advantage, aiding survival as commensals and pathogens of humans by allowing them to evade host immune mechanisms, resisting antifungal treatment, and withstanding the competitive pressure from other microorganisms. Biofilm formation in NCAC species, besides possibly being a key factor for the survival of these species, may also be responsible for them being particularly well adapted to colonization of tissues and indwelling devices.

In the present study, the biofilm formation ability of different clinical isolates of NCAC species was evaluated and the results (Figure II.1) showed that all NCAC species studied (*C. tropicalis*, *C. parapsilosis* and *C. glabrata*), formed biofilms on polystyrene surfaces under the assayed conditions, although to different extents depending on the species and strain. These results were in agreement with those of other authors, who reported that biofilm formation by *Candida* species occurs on a number of abiotic surfaces, including polystyrene (Hawser *et al.,* 1994; Shin *et al.,* 2002; Ramage *et al.,* 2006; Parahitiyawa *et al.,* 2006). Significant statistical differences were found for biofilm production by the NCAC species in SDB medium. In fact, *C. glabrata* strains were, in general, less able to form biofilms than *C. parapsilosis* and *C. tropicalis* strains. These results are in accordance with Shin *et al.* (2002) who reported that biofilm positivity occurred most frequently in isolates of *C. tropicalis*, followed by *C. parapsilosis* and *C. glabrata*.

It was noted that biofilm forming ability of *C. parapsilosis* species was highly strain dependent, which was less evident with both *C. glabrata* and *C. tropicalis*. These observations corroborate previous reports for *C. albicans* whose growth and virulence attributes, together with biofilm formation (Hawser *et al.,* 1994; Jin *et al.,* 2003) have been shown to be

highly strain dependent. Such findings undoubtedly reflect inherent physiological differences between strains and could have significance with respect to pathogenic potential.

Despite the inherently destructive nature of SEM processing, the method provided useful information on biofilm structure and on the different cellular morphologies. It is known that biofilm structure is dependent on environmental factors including growth conditions, nature of colonized surface (Hawser *et al.,* 1994; Chandra *et al.,* 2001; Jain *et al.,* 2007) and importantly from the perspective of this present study, the microbial species and strains involved (Hawser *et al.,* 1994; Chandra *et al.,* 2001; Kuhn *et al.,* 2002; Parahitiyawa *et al.,* 2006; Jain *et al.,* 2007). SEM did indeed reveal structural and morphological differences for the biofilms of the studied NCAC species and strains. Biofilms of *C. glabrata* (Figure II.2 C) presented as a multilayered structure with blastoconidia intimately packed, for some strains, and for others as a biofilm composed of cell clusters. As expected, there was a total absence of pseudohyphae and hyphae since *C. glabrata* is a non-hyphal species. Recently, Zaw *et al.* (2007) also reported that after 48 h, the biofilms of aerobically grown *C. glabrata* generally revealed a multilayer structure packed with blastoconidia devoid of pseudohyphae and hyphae. In the presented study*, C. parapsilosis* strains (Figure II.2 A) yielded a multilayer biofilm structure that was comprised of a dense network of yeasts and pseudohyphae. Although few studies on the biofilm structure of *C. parapsilosis* strains have been reported, Kuhn *et al.* (2002) described that *C. parapsilosis* biofilms consisted of irregular groupings of blastoconidia on a basal blastoconidia layer. Regarding *C. tropicalis*, its biofilms appeared as discontinuous layers of large blastoconidia anchored to the surface, which was in accordance with the findings of Bizerra *et al.* (2008). The latter also reported that *C. tropicalis* biofilms formed in SDB medium, contained only

blastoconidia or generated a multilayer heterogeneous structure covering the entire surface as a thick biofilm. In the present study, large quantities of hyphal elements were found in *C. tropicalis* 12 biofilms (vaginal clinical isolate). It has been suggested (Baillie *et al.,* 1999; Ramage *et al.,* 2002) that the presence of such hyphae may have importance in the structural integrity of multilayered biofilms. The present study reinforces and emphasizes a previous study where one *C. tropicalis* strain formed a thin layer of hyphae (in YNB) compared with other strains only presenting blastoconidia (Kuhn *et al.,* 2002). Parahitiyawa *et al.* (2006), reported that on polystyrene surfaces, *C. tropicalis* biofilms consisted of large coaggregated microcolonies of blastoconidia with a thick extracellular polymeric layer. In fact, almost all microorganisms display structural heterogeneity within their biofilm architecture (Wimpenny *et al.,* 2000). The present work indicates that this heterogeneity appears to be common in biofilms formed by *C. glabrata*, *C. tropicalis* and *C. parapsilosis* strains, revealing new important aspects on NCAC species biofilm ultrastructure.

One of the most important characteristics of both bacterial and fungal biofilms is the presence and composition of the extracellular matrix (Donlan *et al.,* 2002; Al-Fattani *et al.,* 2006). There is a general consensus that the biofilm matrix acts as a barrier to diffusion of antimicrobial agents, thereby limiting access of antimicrobials to organisms at the base of the biofilm (Al-Fattani *et al.,* 2006). In this study, biofilm matrices were analyzed for carbohydrate and protein content (Table II.1). Significantly, consistent differences were found in the composition of biofilms of the NCAC species. Matrices isolated from *C. parapsilosis* biofilms consisted of high amounts of carbohydrates and small amounts of proteins, whilst *C. tropicalis* biofilms were low in both carbohydrate and protein content. These results are in accordance with previous work (Al-Fattani *et al.,* 2006) on *C. tropicalis* biofilm matrices which that they were mainly composed of

hexosamine, with smaller amounts of carbohydrate and proteins. To the authors' knowledge, this is the first report on the analysis of the biofilm matrices of *C. parapsilosis* and *C. glabrata*. Interestingly, the matrices recovered from *C. glabrata* strains had higher amounts of both proteins and carbohydrates. This is an interesting result, especially when related to potential virulence of this species whose infections yield both the highest mortality rate (Kcrmery, 1999) and resistance to antifungal agents (Tsai *et al.,* 1997).

The three different sources (body sites) for the clinical isolates represent very diverse ecological niches and differ in many biotic and abiotic factors. Recent reports have demonstrated that blood isolates produce greater quantities of biofilm compared with oral isolates (Kumar *et al.,* 2006). In this current study, no correlation was found in terms of biofilm forming ability and matrix composition with the origin of the isolate. However, biofilm structure analysis did highlight some interesting aspects. For *C. glabrata*, the biofilm structure for isolates from the same origin did appear to be similar. This was also true for *C. tropicalis* urinary tract isolates. It could readily be hypothesized that for certain body sites, colonization requires a particular phenotype with respect to biofilm formation. Such a biofilm phenotype might be genetically rather than environmentally governed, thus explaining why the biofilm structural differences could be detected in these *in vitro* studies. Through elucidating such inherent differences, it might be possible to identify and specifically combat strains adapted for infection at particular body sites. It must be emphasized, however, that further investigations with isolates from specific environments are required to confirm this hypothesis.

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References

Al-Fattani MA, Douglas LJ (2006). Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* **55**: 999-1008.

Al-Fattani MA, Douglas LJ (2004). Penetration of *Candida* biofilms by antifungal agents. *Antimicrob Agents Ch* **48**: 3291-3297.

Azeredo J, Henriques M, Sillankorva S, *et al.,* (2003). Extraction of exopolymers from biofilms: the protective effect of glutaraldehyde. *Water Sci Technol* **47**: 175-179.

Baillie GS, Douglas LJ (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J Antimicrob Chemother* **46**: 397–403.

Baillie GS, Douglas LJ (1999). Role of dimorphism in the development of *Candida albicans* biofilm. *J Med Microbiol* **48**: 671-679.

Bassetti M, Righi E, Costa A, *et al.,* (2006) Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infect Dis* **6**:21.

Bizerra CF, Nakamura CV, Poersch C, *et al*., (2008) Characteristics of biofilm formation by *Candida tropicalis* and antifungal resistance. *FEMS Yeast Res* **8:** 442-450.

Bonassoli LA, Bertoli M, Svidzinski TIE (2005). High frequency of *Candida parapsilosis* on the hands of healthy hosts. *J Hosp Infect* **59:**159-162

Chandra J, Kuhn DM, Mukherjee PK, *et al*., (2001) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture and drug resistance. *J Bacteriol* **183**: 5385-5394.

Costerton JW, Lewandowski Z, Caldwell DE, *et al.,* (1995). Microbial biofilms. *Annu Rev Microbial* **49**: 711-745.

Donlan RM, Costerton JW (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**: 167-193.

Dubois M, Gilles KA, Hamilton JK, *et al.,* (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**, 350-356.

Fanello S, Bouchara JP, Jousset N, *et al.,* (2001). Nosocomial *Candida albicans* acquisition in a geriatric unit: epidemiology and evidence for person-to-person transmission. *J Hosp Infect* **47**: 46–52.

Fridkin SK, Jarvis WR (1996). Epidemiology of nosocomial fungal infections. *Clin Microbiol Rev* **9**: 449-511.

Hajjeh RA, Sofair AN, Harrison LH, *et al.* (2004). Incidence of bloodstream infections due to *Candida* species and *in vitro* susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J Clin Microbiol* 2004; **42**:1519–1527.

Hargety JA, Ortiz J, Reich D, *et al.,* (2003). Fungal infections in solid organ transplant patients. *Surg Infect (Larchmt)* **4**: 263-271.

Hawser SP, Douglas LJ (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infect Immun* **62**: 915-921.

Hawser SP, Douglas LJ (1995). Resistance of *Candida albicans* biofilms to antifungal agents in vitro. *Antimcrob Agents Ch* **39**:2128-2131.

Jain N, Kohli R, Cook E, *et al*. (2007) Biofilm formation by and antifungal susceptibility of *Candida* isolates from urine. *Appl Environ Microbiol* **73**: 1693-1703.

Jin Y, Yip KH, Samaranayake YH, *et al.,* (2003). Biofilm-forming ability of *Candida albicans* is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. *J Clin Microbiol* **41**: 2961-2967.

Kcrmery K (1999). *Torulopsis glabrata* an emerging pathogen in cancer patients. *Int J Antimicrob Agents* **11**: 1-6.

Kiehn TE, Edwards FF, Armstrong D (1980). The prevalence of yeasts in clinical specimens from cancer patients. *Am J Clin Pathol* **73**:518–521.

Kojic EM, Darouiche RO (2004). *Candida* infections of medical devices. *Clin Microbiol Rev* **17**: 255-267.

Kuhn DM, Chandra J, Mukherjee PK, *et al.,* (2002). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun* **70**: 878-888.

Kumamoto CA (2002). *Candida* biofilms. *Curr Opin Microbiol* 2002; **5**: 608-611.

Kumar CPG, Menon T (2006). Biofilm production by clinical isolates of *Candida* species. *Medi Mycol* **44**: 99-101.

Mukherjee PK, Chandra J (2004). *Candida* biofilm resistance. *Drug Resist update* **7**: 301-309.

Nucci M, Colombo AL (2007). Candidemia due to *Candida tropicalis*: clinical, epidemiologic and microbiologic characteristics of 188 episodes occurring in tertiary care hospitals. *Diagn Microbiol Infect Dis* **58**: 77–82.

Odds FC. *Candida* and Candidosis. 2nd edn. Bailliere Tindall, London, England, 1998.

Parahitiyawa NB, Samaranayake YH, Samaranayake LP, *et al*., (2006) Interspecies variation in *Candida* biofilm formation studied using the Calgary biofilm device. *APMIS* **114**: 298–306

Ramage G, Martinez JP, Lopez-Ribot JL (2006). *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res* **6**: 979-986.

Ramage G, VandeWalle K, Bachmann SP, *et al.,* (2002). *In vitro* pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. *Antimicrob Agents Ch* **46**: 3634-3636.

Rho J, Shin JH, Song JW, *et al.,* (2004) Molecular investigation of two consecutive nosocomial clusters of *Candida tropicalis* candiduria using pulsed-field gel electrophoresis. *J Microbiol* **42**: 80–86.

Samaranayake LP, Fidel PL, Naglik JR, *et al.,* (2002). Fungal infections associated with HIV infection. *Oral Dis* **8**: 151-160.

Samaranayake YH, Ye J, Yau JYY, *et al.,* (2005). *In vitro* method to study antifungal perfusion in *Candida* biofilms. *J Clin Microbiol* **43**: 818-825.

Scherer S, Stevens DA (1987). Application of DNA typing methods to epidemiology and taxonomy of *Candida* species. *J Clin Microbiol* **25**: 675–679.

Shin JH, Kee SJ, Shin MG, *et al*., (2002). Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. *J Clin Microbiol* **40**: 1244-1248.

Tsai H, Bobek LA (1997). Studies of the mechanism of human salivary histatin-5 candidacidal activity with histatin-5 variants and azole-sensitive and -resistant *Candida* species. *Antimicrob Agents Ch* **41**:2224–2228

Weinberger M, Leibovici L, Perez S, *et al.,* (2005). Characteristics of candidaemia with *Candida albicans* compared with non *albicans Candida* species and predictors of mortality. *J Hosp Infect* **61**: 146–154.

Wimpenny J, Manz W, Szewzyk U (2000). Heterogeneity in biofilms. *FEMS Microbiol Rev* **24**: 661-671.

Zaw MT, Samaranayake YH, Samaranayake LP (2007). In vitro biofilm formation of *Candida albicans* and non-*albicans Candida* species under dynamic and anaerobic conditions. *Arch Oral Biol* **52***:* 761-767.

Chapter III

In vitro biofilm activity of non-*Candida albicans* *Candida* species

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Abstract

Candidosis have been attributed to *C. albicans*, however infections caused by non-*Candida albicans Candida* (NCAC) species are increasingly being recognized. The ability of *Candida* to grow as a biofilm is an important feature that promotes both infection and persistence in the host. The biofilm` activity is significant since high activity might be associated with enhanced expression of putative virulence factors, whilst in contrast low activity has previously been suggested as a mechanism for resistance of biofilm cells to antimicrobials. The aim of this study was to determine the metabolic activity of *in vitro* biofilms formed by different clinical isolates of NCAC species.

The *in situ* total metabolic activity of *C. parapsilosis*, *C. tropicalis* and *C. glabrata* biofilms was determined using 2, 3-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay, and the number of cultivable cells was also established by CFU (colony forming unit) counts. The biofilm structure was assessed by scanning electron microscopy (SEM).

Results showed that total biofilm metabolic activity was species and strain dependent. *Candida glabrata* exhibited the lowest biofilm metabolic activity despite having the highest number of biofilm cultivable cells. Similarly, the metabolic activity of resuspended *C. glabrata* biofilm and planktonic cells was lower than that of the other species. This study demonstrates the existence of intrinsic activity differences amongst NCAC species, which could have important implications in terms of species relative virulence. Furthermore, the absence of an obvious correlation, between cultivable cells number and total biofilm activity raises the question about which parameter is the most appropriate for the *in vitro* assessment of biofilms and their potential clinical significance.

Keywords: non-*Candida albicans Candida* species; biofilm activity, XTT reduction; cultivable cells

Introduction

Candidosis is the most prevalent opportunistic fungal infection of humans and of particular significance into patients undergoing treatment for cancer (Kiehn *et al.,* 1980), organ transplantation (Hargety *et al.,* 2003) and receiving broad-spectrum antibiotics (Samaranayake, 1990). Candidosis is also one of the most common and persistent infections in HIV-infected individuals and AIDS patients (Samaranayake *et al.,* 2002).

Amongst *Candida* species, *Candida albicans* is the most commonly isolated and responsible for the majority of superficial and systemic infections (Odds, 1994). However, many non-*Candida albicans Candida* (NCAC) species, such as *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis* have recently emerged as important pathogens in suitably debilitated individuals. This fact is highlighted by recent epidemiology studies reporting that NCAC are currently estimated to be responsible for approximately 60% of fungemia (Colombo *et al.,* 2003; Bassetti *et al.,* 2006).

A major virulence factor of *Candida* is its ability to adapt to a variety of different habitats and the consequent formation of surface-attached microbial communities known as biofilms (Costerton *et al.,* 1995; Douglas, 2003). *Candida* biofilms can develop on natural host surfaces or on biomaterials used in indwelling medical devices. Importantly, such biofilms are phenotypically distinct from their 'free-living' or planktonic forms, with biofilms exhibiting elevated resistance to host defences and administered antimicrobial agents (Baillie *et al.,* 2000; Samaranayake *et al.,* 2002; Hasan *et al.,* 2009). The clinical significance of biofilms is highlighted by recent estimates that over 65% of all hospital infections originate from these microbial communities (Mah *et al.,* 2001). As a consequence researchers are now recognising the importance of studying biofilm communities

rather than planktonic forms when characterising the pathogenic potential of microorganisms. So far, a variety of methods have been described for both the *in vitro* production of biofilms and their subsequent characterization. Indirect methods based on 96-well microtiter plate assays, which allow the simultaneous quantification of yeasts in a large number of biofilm samples have been recommended due to their simplicity and sensitivity (Hawser, 1996; Kuhn *et al.,* 2002). A frequently used approach to quantify biofilm cells activity has been the reduction assay of the tetrazolium salt 2, 3-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT). In this colorimetric method, XTT is reduced to an XTT formazan product by mitochondrial dehydrogenases of metabolically active cells. The resulting colorimetric changes are considered to be proportional to the number of living cells and their metabolic activity (Hawser, 1996; Kuhn *et al.,* 2002).

Previous *Candida* biofilm studies have been mainly focused on *C. albicans* biofilms (Baillie *et al.,* 1999; Hawser, 1996; Chandra *et al.,* 2001; Jin *et al.,* 2003; Henriques *et al.,* 2006), with very few examining NCAC species (Hawser *et al.,* 1994; Shin *et al.,* 2002; Al-Fattani *et al.,* 2006). Furthermore, the vast majority of candidal biofilm research has been focussed on the assessment of biofilm development either by biomass determination or by quantifying the number of cells in the biofilm. The activity of the biofilm itself or its cellular composition has rarely been investigated (Kuhn *et al.,* 2002). This oversight is important given the fact that biofilm activity has significance in terms of pathogenicity as it is a likely indicator of growth, production of hydrolytic enzymes and, in the case of reduced activity, of possible resistance to antimicrobial activity. Therefore, considering that the biofilm activity is a requirement in *Candida* infections, the aim of this study was to determine the metabolic activity of *in vitro* biofilms formed by different clinical isolates of NCAC species.

Materials and methods

Organisms and growth conditions

A total of 18 clinical strains (Table III.1) of *C. parapsilosis* (n=6), *C. tropicalis* (n=6) and *C. glabrata* (n=6) that had previously been recovered from oral, vaginal or urinary tract infections, were used in this study. Strains isolated from vaginal and urinary tract infections were kindly provided by the Hospital of São Marcos (Braga, Portugal). *Candida tropicalis* strains 12 and 75 (recovered from the vaginal tract) were provided by the University of Maringá (Maringá, Brazil). Oral isolates were originally isolated from patients attending the Clínica dos Congregados (Braga, Portugal). Three reference strains, namely *C. parapsilosis* (ATCC 22019), *C. tropicalis* (ATCC 750) and *C. glabrata* (ATCC 2001) were also included in this study. The identity of all isolates was confirmed using CHROMagar® *Candida* (CHROMagar, Paris, France) and by PCR sequencing using specific primers (ITS1 and ITS4) targeting the 5.8S ribosomal RNA subunit gene (Williams *et al.,* 1995).

Before the experiments, all isolates were cultured on sabouraud dextrose agar (SDA; Merck, Germany) for 48 h at 37°C. Cells were then subcultured in sabouraud dextrose broth (SDB; Merck, Germany) for 18 h at 37°C in an orbital shaker (120 rev/min). Cells were then harvested by centrifugation at 3000 x *g* for 10 min at 4°C and washed twice in phosphate buffer saline (PBS pH 7, 0.1 M). The cell pellets were finally resuspended in SDB and the cell concentration was adjusted using an improved Neubauer haemocytometer to a standardised level (1×10^7 or 1×10^8 cells ml^{-1}) depending on the experiment.

Biofilm formation

Biofilms were produced in 96-wells microtiter plates (Orange Scientific, Braine-l' Alleud, Belgium). Briefly, standardised inocula (200 µl of yeast cell suspension containing $1x10^7$ cells ml^{-1} in SDB) were placed into selected wells and incubated at 37°C in an orbital shaker incubator at 120 rev/min. After 24 h, an aliquot of 100 µl of SDB medium was removed and an equal volume of fresh SDB added. The microtiter plates were then incubated for further 48 h. Experiments were performed in triplicate and on at least three separate occasions.

Biofilm characterization

In situ biofilm metabolic activity

An XTT reduction assay (Hawser, 1996, 1998) was used to determine the *in situ* biofilm metabolic activity of the NCAC strains. After biofilm formation, the culture medium was aspirated and the non-adherent cells were removed by washing the biofilms twice in PBS. A 200 µl aliquot of a solution containing 100 µg/µl of XTT (2, 3-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Sigma-Aldrich, USA) and 10 µg $µl^{-1}$ of PMS (phenazine methosulfate) (Sigma-Aldrich, USA) was then added to each pre-washed biofilm and also to the control wells (to measure background XTT levels). The plates were then incubated for 3 h in the dark, at 37°C with agitation (120 rev/min). Colorimetric changes were measured at 490 nm using a microtiter plate reader (Bio-Tek® Synergy HT, Izasa, Portugal) and the absorbance values were standardised per unit area of well (absorbance/cm^2).

Quantification of biofilm cultivable cells

The number of cultivable biofilm cells was determined by counting colony forming units (CFUs) following biofilm cells resuspension. Briefly, biofilms were first washed twice in PBS to remove loosely attached cells and the biofilm was then resuspended by repeated pipetting. Complete removal of the biofilm was confirmed by subsequent crystal violet staining and spectrophotometric reading for inspection of the wells. The resuspended biofilm (500 µl) was vigorously vortexed for 5 min to disrupt the biofilm matrix and serial decimal dilutions (in PBS) were plated onto SDA. Agar plates were incubated for 24 h at 37°C, and the total CFUs per unit area (Log CFU/cm^2) of microtiter plate well were enumerated. Experiments were repeated on three occasions with individual samples evaluated in triplicate.

Biofilm structure

Biofilm structure was examined by Scanning Electron Microscopy (SEM). Biofilms were formed by seeding 2 ml of the standardized cell suspension ($1x10^7$ cells ml^{-1} in SDB) into 24-well plates (Orange Scientific, Braine-l`Alleud, Belgium) and incubated for 48 h at 37°C and 120 rev/min. After 24 h, 1 ml of SDB medium was removed and an equal volume of fresh SDB added. At 48 h, the medium was aspirated and non-adherent cells removed by washing the biofilms twice with sterile ultra-pure water. Samples were dehydrated with alcohol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. Samples were kept in a desiccator until the base of the wells was removed for analysis. Prior to observation, the base of the wells were mounted onto aluminium stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

Quantification of cellular metabolic activity

Metabolic activity of biofilms cells

Biofilms were removed from the microtiter plate wells as described previously and the cell concentration adjusted to 1×10^8 cells ml^{-1} using an improved Neubauer haemocytometer. Cells from 1 ml of the standardised cell preparation by centrifugation (3000 x *g*), and the supernatant was discarded. An aliquot of 1 ml of XTT solution (100 µg $µl^{-1}$ of XTT and 10 µg $µl^{-1}$ of PMS) was added to the yeast cells pellet. The mixture was then incubated in the dark for 3 h at 37°C and 120 rev/min. Colorimetric changes were measured as previously described and activity expressed as absorbance/$1x10^8$ cells. Experiments were repeated on three occasions with individual samples evaluated in triplicate.

Metabolic activity of planktonic cells

Cells were inoculated in SDB for 18 h at 37°C under agitation (120 rev/min) and then harvested by centrifugation (3000 x *g* for 10 min at 4°C) and washed twice in PBS. After resuspension in PBS, the cell concentration was adjusted to 1 x 10^8 cells ml^{-1} and the cellular metabolic activity was measured as previously described for the resuspended biofilm cells.

Statistical analysis

Results were compared using a one-way analysis of variance (ANOVA) by applying Levene´s test of homogeneity of variance and the Tukey multiple-comparisons test, using SPSS software (SPSS [Statistical Package for the Social Sciences], Inc., Chicago, IL). All tests were performed with a confidence level of 95%.

Results and discussion

The primary aim of this study was to determine the total *in situ* biofilm activity of 21 strains of NCAC species using an XTT reduction assay (Figure III.1).

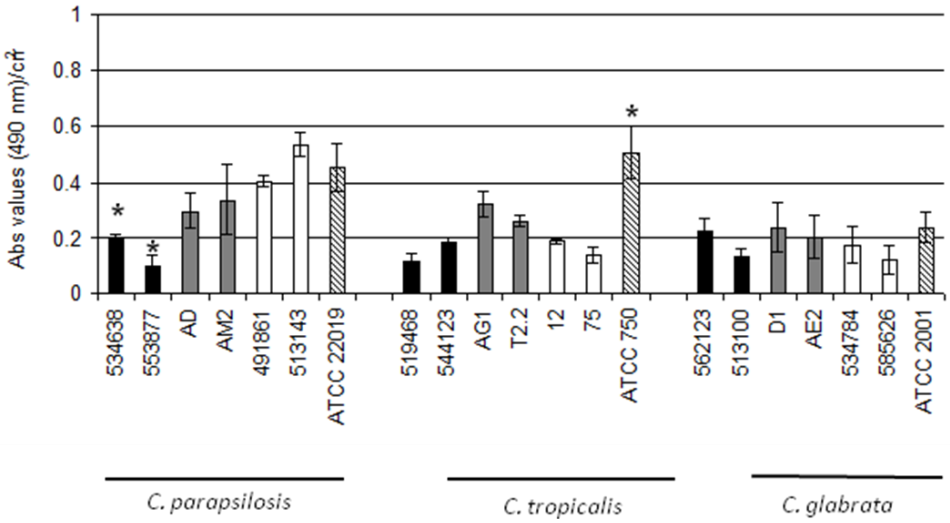

Figure III.1 Absorbance values of XTT solutions per cm^2 of biofilms formed in SDB for 48 h by different clinical isolates of *C. parapsilosis*, *C. tropicalis* and *C. glabrata*. Error bars represent standard deviation. *Strains of the same species that are significantly different (P<0.05).

It was clearly evident that biofilm metabolic activity was species and strain dependent. Specifically, the overall mean metabolic activity of *C. glabrata* (Abs/cm^2 = 0.19±0.05) biofilms was lower than for *C. parapsilosis* (Abs/cm^2 = 0.33±0.15) and *C. tropicalis* (Abs/cm^2 = 0.24±0.14). Such intra species variation in terms of biofilm formation has previously been demonstrated albeit using parameters other than activity (Hawser, 1994; Shin *et al.,* 2002). It was interesting to note that in the case of *C.*

parapsilosis, both urinary tract isolates (534638 and 553877) had significantly (P<0.05) lower biofilm metabolic activity (Abs/cm^2 = 0.20±0.01; Abs/cm^2 = 0.09±0.04) compared with the other *C. parapsilosis* strains. Whether this finding relates to an inherent difference between strains from distinct pathological origins is unclear, and further investigation involving larger number of strains from these conditions is needed to clarify this point.

There are several possible reasons for the observed species and strain differences in *in situ* biofilm metabolic activity. A key factor would obviously be the relative number of metabolic active cells within the biofilm itself. In order to analyse this, CFU counts for each biofilm were determined. In contrast to what would perhaps be expected, there was no correlation between biofilm metabolic activity (Figure III.1) and CFU number (Figure III.2).

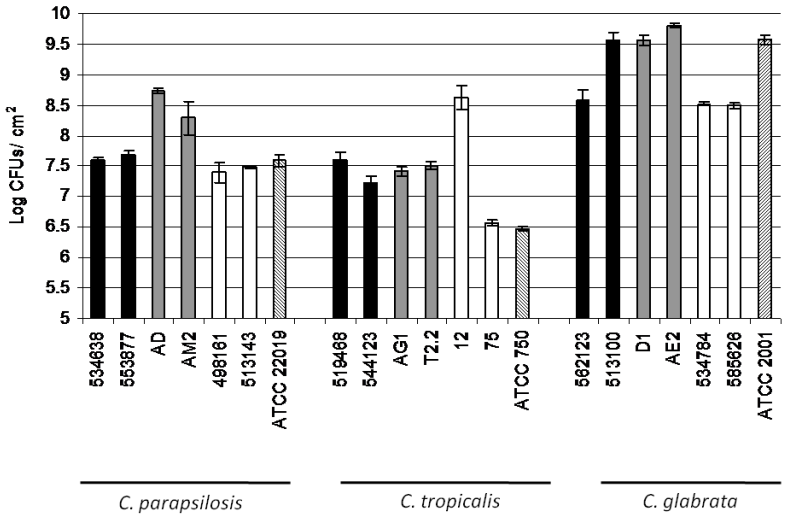

Figure III.2 Logarithm of number cells of *C. parapsilosis*, *C. tropicalis* and *C. glabrata* per cm^2 the biofilm formed in SDB after 48 h. Error bars represent standard deviation. *Strains of the same species that are significantly different (P<0.05).

It was evident that despite *C. glabrata* biofilms having the lowest metabolic activity, these biofilms had significantly (P>0.05) higher number of cultivable cells per unit area (Log CFU/cm^2 = 9.16±0.59) when compared with *C. tropicalis* (Log CFU/cm^2 = 7.34±0.72) and *C. parapsilosis* (Log CFU/cm^2 = 7.82±0.09). Furthermore, also the intra-strain variation observed for *C. parapsilosis* and *C. tropicalis* in terms of biofilm metabolic activity (Figure III.1) was not associated with differences in CFU numbers (Figure III.2).

The observed discrepancy between *in situ* total biofilm activity and the number of cultivable cells does have important implications for biofilm studies, since it is often assumed that metabolic activity measurements provide a sufficient correlation to indirectly quantify biofilms (Hawser, 1996; Chandra *et al.,* 2001; Zaw *et al.,* 2007). This situation may be adequate when comparing a single strain type under different environmental conditions but is problematic when multiple strains and species are being compared (Kuhn *et al.,* 2003).

In terms of species and strain differences, consideration has to be given to inherent differences in the relative size, morphology, and biochemistry of cells. It is known that *C. glabrata* cells are generally smaller (1-4 μm) than *C. tropicalis* (4-8 μm) and *C. parapsilosis* (2.5-4 μm) (Odds, 1998) and also have a narrower spectrum of carbohydrate utilisation (Kwon-Chung, *et al.,* 1992; Fidel *et al.,* 1999). Unlike *C. parapsilosis* and *C. tropicalis*, *C. glabrata* (Figure III.3) is unable to generate filamentous forms which may also be expected to exhibit different metabolic activity, and thus contribute to species differences (Kuhn *et al.,* 2002). Inherent species differences would be supported by the control experiments (Table III.1) where planktonic cultures illustrated similar differences between the species with *C. glabrata* again having lower cellular metabolic activity compared with the other two species.

Table III.1 Non-*Candida albicans Candida* strains used in this study, values of *in situ* total biofilm activity, relative biofilm activity and metabolic activity of biofilm and planktonic cells

Species	Strains	Origin	***In situ* total biofilm activity** (Abs values/cm^2) ± SD	**Relative biofilm activity** (Abs values/10^8 CFU)	**Cellular metabolic activity** (Abs values/10^8 cells) ± SD	
					Biofilm cells	**Planktonic cells**
C. parapsilosis	534638	Urinary tract	0.198 ± 0.01	0.518	0.548 ± 0.08	0.621 ± 0.05
	553877		0.099 ± 0.04	0.199	0.323 ± 0.04	0.474 ± 0.13
	AD	Oral tract	0.297 ± 0.07	0.055	0.387 ± 0.06	0.702 ± 0.19
	AM2		0.337 ± 0.13	0.116	0.587 ± 0.09	0.407 ± 0.09
	491861	Vaginal	0.405 ± 0.02	1.311	0.534 ± 0.11	0.554 ± 0.11
	513143		0.537± 0.04	1.677	0.496 ± 0.11	0.669 ± 0.11
	ATCC 22019	Reference	0.454 ± 0.09	1.134	0.221 ± 0.05	0.492 ± 0.05
C. tropicalis	519468	Urinary tract	0.115 ± 0.03	0.295	0.273 ± 0.01	0.702 ± 0.15
	544123		0.179 ± 0.02	1.059	0.283 ± 0.07	0.867 ± 0.07
	AG1	Oral tract	0.322 ± 0.05	1.094	0.345 ± 0.07	0.467 ± 0.04
	T2.2		0.259 ± 0.02	0.922	0.162 ± 0.02	0.585 ± 0.09
	12	Vaginal	0.185 ± 0.01	0.041	0.313 ± 0.02	0.298 ± 0.01
	75		0.137 ± 0.03	3.672	0.376 ± 0.07	0.323 ± 0.01
	ATCC 750	Reference	0.507 ± 0.09	16.80	0.665 ± 0.11	0.688 ± 0.02
C. glabrata	562123	Urinary tract	0.223 ± 0.04	0.006	0.228 ± 0.07	0.281 ± 0.09
	513100		0.129 ± 0.03	0.002	0.100 ± 0.02	0.149 ± 0.01
	D1	Oral tract	0.237 ± 0.09	0.006	0.179 ± 0.03	0.138 ± 0.07
	AE2		0.201± 0.08	0.052	0.167 ± 0.02	0.135 ± 0.04
	534784	Vaginal	0.172 ± 0.07	0.049	0.131 ± 0.03	0.079 ± 0.03
	585626		0.118 ± 0.05	0.031	0.084 ± 0.02	0.201 ± 0.06
	ATCC 2001	Reference	0.238 ± 0.06	0.084	0.181 ± 0.05	0.732 ± 0.14

Specifically, it was shown that *C. glabrata* biofilm cells had, on average, a lower metabolic activity (Abs/1x10^8cells = 0.15±0.03) than either *C. tropicalis* (Abs/1x10^8cells=0.35±0.16) or *C. parapsilosis* (Abs/1x10^8cells=0.45±0.15) cells (Table III.1). There were, however, some discrepancies when comparing the relative strain hierarchy for metabolic activity for the planktonic and biofilm cultures. A possible explanation for this relates to potential additional significant phenotypic differences occurring between the two lifestyles (Mukherjee *et al.,* 2003; Ramage *et al.,* 2005). Perhaps the most important of these, are those factors that relate to the promotion, persistence and virulence of the organisms within the host environment.

As biofilm cells are organised into structured communities embedded within an extracellular matrix, activity within biofilms would be dependent on nutrient access and availability of oxygen, together with removal of waste products. These factors may vary because of inherent differences in the biofilms produced by the tested strains and species, with the resulting alteration in cellular and biofilm metabolic activity. Indeed, evidences of variation in biofilm spatial arrangement is possible observed in Figure III.3.

For instances (Figure III.3B), concerning *C. tropicalis* 12, which possess high number of CFU (Figure III.2) and low activity (Figure III.1), it is possible to verify that its biofilm is more cohesive than *C. tropicalis* 75, which presents lower CFU number (Figure III.2) as well as metabolic activity (Figure III.1). The same was observed for *C. glabrata* (Figure 3III.A) and *C. parapsilosis* (Figure III.3C) biofilms. Thus, an effect on the ability of XTT to diffuse into these 'different' biofilms is an aspect that also cannot be disregarded. When total biofilm activity was normalised against 1x10^8 CFU of resuspended biofilm (Table III.1), it was evident that the relative biofilm activity of *C. glabrata* was 100 times less than *C. tropicalis* and 10 times lower than *C. parapsilosis*.

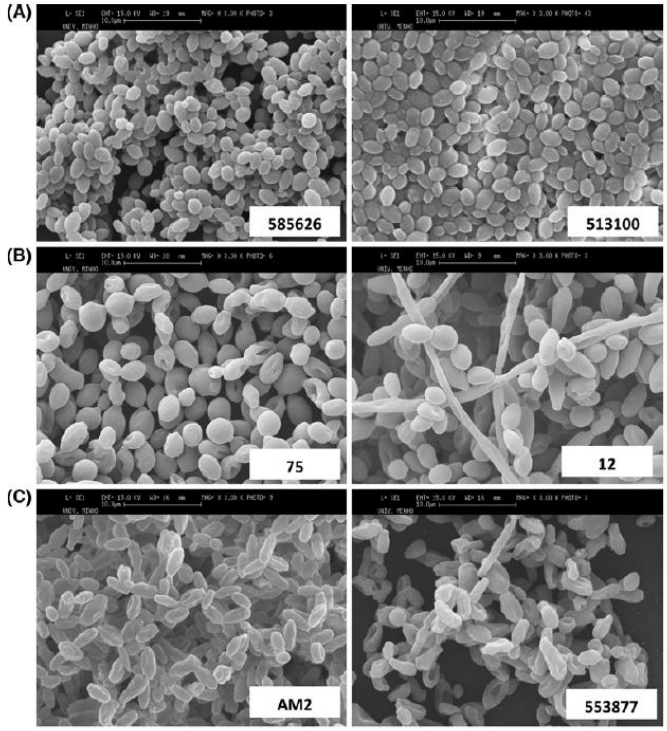

Figure III.3 Biofilm scanning electron microscopy images of two *C. glabrata* **(A)**, *C. tropicalis* **(B)** and *C. parapsilosis* **(C)** strains formed in SDB for 48 h. Magnification 3000 x, bar 10 µm.

Interestingly, this analysis highlights additional heterogeneity in terms of relative biofilm metabolic activity between *C. parapsilosis* and *C. tropicalis*. Furthermore, the results indicate that, in general, biofilms formed by clinical isolates had a much lower relative activity compared to the corresponding reference strains, with the exception of *C. parapsilosis* ATCC 22019.

In summary, this work underlines both species and strain differences in biofilm metabolic activity which in part reflects the inherent physiological differences between the organisms. However, it has also to be considered that intrinsic biofilm factors may have a direct effect on the

responses obtained. These findings may have significance concerning the pathogenic potential of the strains tested when extrapolated to *in vivo* situations, with the clinical isolates generally having a lower activity than the equivalent reference species. Such a relatively low activity could promote persistence of the isolates within a clinical environment once the biofilm has become established. Another important aspect raised by this study is that future research needs to consider which is the most appropriate parameter to investigate *in vitro* biofilm models since total biofilm activity or cultivable cells number do not necessarily reflect the behaviour of the biofilm itself.

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References

Al-Fattani MA, Douglas LJ (2006). Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* **55**: 999-1008

Baillie GS, Douglas LJ (1999). Role of dimorphism in the development of *Candida albicans* biofilm. *J Med Microbiol* **48**: 671-679

Baillie GS, Douglas LJ (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J Antimicrob Chemother* **46**: 397–403

Bassetti M, Righi E, Costa A, *et al*., (2006). Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infect Dis* **6**: 21

Chandra J, Kuhn DM, Mukherjee PK, *et al.,* (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture and drug resistance. *J Bacteriol* **183**: 5385-5394.

Colombo AL, Perfect J, DiNubile M, *et al.,* (2003) Global distribution and outcomes for *Candida* species causing invasive candidiasis: results from an international randomized double-blind study of caspofungin versus amphotericin B for the treatment of invasive candidiasis. *Eur J Microbiol Infect Dis* **22**: 470-474

Costerton JW, Lewandowski Z, Caldwell DE, *et al.,* (1995). Microbial biofilms. *Annu Rev Microbial* **49**: 711-745

Douglas LJ (2003). *Candida* biofilms and their role in infection. *Trends Microbiol* **11**: 30-36.

Fidel PL, Vazquez JA, Sobel JD (1999). *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin Microbiol Rev* **12**: 80-96

Hargety JA, Ortiz J, Reich D, *et al.,* (2003). Fungal infections in solid organ transplant patients. Surg Infect (Larchmt) **4**: 263-271

Hasan F, Xess I, Wang X, *et al.,* (2009). Biofilm formation in clinical *Candida* isolates and its association with virulence. *Microbes Infect* **11**: 753-761

Hawser SP, Douglas LJ (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infect Immun* **62**: 915-921

Hawser SP (1996). Comparisons of the susceptibility of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronized *C. albicans* cells. *J Med Vet Mycol* **34**: 149-152

Hawser SP (1998). Production of extracellular matrix by *Candida albicans* biofil*ms. J Med Microbiol* **47**: 253-256

Henriques M, Azeredo J, Oliveira R (2006). *Candida albicans* and *Candida dubliniensis*: comparison of biofilm formation in terms of biomass and activity. *Brit J Biomed Sci* **63**: 5-11

Jin Y, Yip KH, Samaranayake YH, *et al.,* (2003). Biofilm-forming ability of *Candida albicans* is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. *J Clin Microbiol* **41**: 2961-2967

Kiehn TE, Edwards FF, Armstrong D (1980). The prevalence of yeasts in clinical specimens from cancer patients. *Amer J Clin Pathol* **73**: 518-521

Kuhn DM, Chandra J, Mukherjee PK, *et al*., (2002). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immunol* **70**: 878-888

Kuhn DM, Balkis M, Chandra J, *et al.,* (2003). Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. *J Clin Microbiol* **41**: 506-508

Kwon-Chung KJ, Bennett JE. Dermatophytoses. In: *Medical Mycology*. 1 ed. Pennsylvania: Lea & Febiger, 1992; 105-161

Mah TFC, Toole GAO (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**: 34-39

Mukherjee PK, Chandra J, Kuhn DM, *et al.,* (2003) Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun* **71**: 4333-4340

Odds FC (1994). Pathogenesis of *Candida* infections. *J Am Acad Dermatol* **31**:2-5

Odds FC. *Candida* and Candidosis, 2n edn. London, Bailliere Tindall, 1998

Ramage G, Saville PS, Thomas PD, *et al*., (2005). *Candida* Biofilms: an Update. *Eukaryot Cell* **4**: 633-638

Samaranayake LP. Host factors and oral candidosis. In: Oral candidosis. London: Butterworth &Co (Publishers), 1990; 66-103

Samaranayake LP, Fidel PL, Naglik JR, *et al.,* (2002). Fungal infections associated with HIV infection. *Oral Dis* **8**: 151-160

Shin JH, Kee SJ, Shin MG, *et al.,* (2002). Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. *J Clin Microbiol* **40**: 1244-1248

Williams DW, Wilson M J, Lewis MAO, *et al*., (1995). Identification o*f Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol* **33**: 2476-2479

Zaw MT, Samaranayake YH, Samaranayake LP (2007). *In vitro* biofilm formation of Candida albicans and non-*albicans Candida* species under dynamic and anaerobic conditions. *Arch Oral Biol* **52**: 761-767

Chapter IV

Silicone colonization by non-*Candida albicans* *Candida* species in the presence of urine

The work presented in this chapter was published:

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Abstract

Urinary tract infections (UTIs) are the most common nosocomial infections and 80% are related to the use of urinary catheters. Furthermore, *Candida* species are responsible for around 15% of UTIs and an increasing involvement of non-*Candida albicans Candida* (NCAC) species (*e.g. C. glabrata*, *C. tropicalis* and *C. parapsilosis*) has been recognised. Given the fact that silicone is frequently used in the manufacture of urinary catheters, the aim of this work was to compare both the adhesion and biofilm formation on silicone of different urinary clinical isolates of NCAC species (*i. e. C. glabrata*, *C. tropicalis* and *C. parapsilosis*) in the presence of urine. Several clinical isolates of NCAC species recovered from patients with UTIs, together with reference strains of each species, were examined. Adhesion and biofilm formation were performed in artificial urine and the biofilm biomass assessed by crystal violet staining. Hydrophobicity and surface charge of cells was determined by measuring contact angles and zeta potential, respectively. The number of viable cells in biofilms was determined by enumeration of CFU after appropriate culture. The biofilm structure was also examined by confocal laser scanning microscopy (CLSM). The results showed that all isolates adhered to silicone in a species- and strain-dependent manner with *C. parapsilosis* showing the lowest and *C. glabrata* the highest levels of adhesion. However, these differences in adhesion abilities cannot be correlated with surface properties since all strains examined were hydrophilic and exhibited a similar zeta potential. Despite a higher number of cultivable cells being recovered after 72 h of incubation, stronger biofilm formation was not observed and CLSM showed an absence of extracellular polymeric material for all isolates examined. In summary, this work demonstrates that all tested NCAC species were able to adhere to and survive on silicone in the presence of urine. Furthermore, *C. glabrata* strains presented higher colonization abilities compared with *C. tropicalis* and *C. parapsilosis* strains, a fact that might explain the larger role of *C. glabrata* colonization and disseminated infections in hospitalized and catheterized patients.

Keywords: urinary tract infections; silicone; urine; non-*Candida albicans Candida species*, adhesion, biofilm

Introduction

Nosocomial infections constitute a serious public health problem, as they are a major cause of morbidity and mortality, and cause an increased time of hospitalization with associated enhanced healthcare costs (Tamura *et al.,* 2003). Urinary tract infection (UTI) is one of the most common types of nosocomial infections (Schaberg *et al.,* 1991, Tamura *et al.,* 2003) and more than 80% of nosocomial UTIs are related to the use of medical devices such as urinary catheters (Febré *et al.,* 1999; Harris *et al,* 1999; Douglas, 2002; Chow *et al.,* 2008). Furthermore, *Candida* species are responsible for around 80% of fungal infections in the hospital environment and 10-15% of UTIs are caused by these microorganisms (Amer *et al.,* 2004). Although, the majority of infections are caused by *Candida albicans* (Richards *et al.,* 1999; Sobel *et al.,* 2000), non-*Candida albicans Candida* (NCAC) species including *Candida glabrata* (Harris *et al.,* 1999; Manzano-Gayosso *et al.,* 2008), *Candida tropicalis* (Kauffman *et al.,* 2000) and *Candida parapsilosis* (Ruan *et al.,* 2008) are emerging as important nosocomial pathogens with a predilection for the urinary tract.

Candida species are commensal microorganisms that become pathogenic when the defence mechanisms of the host is weakened, and these organisms then have the ability to cause a variety of superficial and systemic infections (Kojic *et al.,* 2004). The primary event in *Candida* infection is the adherence of the microorganism to a host and/or medical device surface, often leading to the formation of biofilms (Crump *et al.,* 2000; Chandra *et al.,* 2001a). Once established, *Candida* biofilms serve as a persistent reservoir of infection and, in addition, offer great tolerance to antifungal agents (Chandra *et al.,* 2001b; Ramage *et al.,* 2001; Samaranayake *et al.,* 2005).

Predisposition to the development of fungal infections is mediated by multiple factors (Douglas, 2003) and it is now recognized that implanted devices have a profound clinical impact due to *Candida*-associated biofilms (Douglas, 2002). Thus, the main aim of this work was to compare both the adhesion and biofilm formation ability of different urinary clinical isolates of NCAC species (*i.e. C. glabrata*, *C. tropicalis* and *C. parapsilosis*) onto silicone in the presence of urine.

Materials and methods

Organisms

A total of six urinary isolates of *C. parapsilosis* (534638 and 553877), *C. tropicalis* (519468 and 544123) and *C. glabrata* (562123 and 513100) were used in the course of this study. Three reference strains, namely *C. parapsilosis* (ATCC 22019), *C. tropicalis* (ATCC 750) and *C. glabrata* (ATCC 2001) were also examined. Strains isolated from UTIs were gifted from the collection of the Hospital of São Marcos, (Braga, Portugal). The identity of all isolates was confirmed using CHROMagar *Candida* and by PCR sequencing using specific primers (ITS1 and ITS4) targeting 5.8S rRNA subunit gene (Williams *et al.,* 1995). Genomic DNA was extracted according to previously described procedures (Scherer *et al.,* 1987). PCR products were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Perkin Elmer, Applied Biosystems).

Medium and growth conditions

All *Candida* isolates were subcultured on sabouraud dextrose agar (SDA; Merck) for 48 h at 37°C, followed by culture in sabouraud dextrose broth (SDB, Merck) for 18 h at 37°C in an orbital shaker (120 rev/min). Cells were then harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with PBS (pH 7, 0.1 M). The cell pellets were finally resuspended in artificial urine (AU) and cell concentration was adjusted using an improved Neubauer haemocytometer to 1×10^7 cells ml^{-1}. AU (pH 5.8) was prepared with slight modification to that previously described (Shin *et al.,* 2002; Jain *et al.,* 2007). The composition of the AU used in this study was, $CaCl_2$ (0.65 g l^{-1}), $MgCl_2$ (0.65 g l^{-1}), NaCl (4.6 g l^{-1}), Na_2SO_4 (2.3 g l^{-1}), $Na_3C_3H_5O(CO_2)_3$ (0.65 g l^{-1}), $Na_2C_2O_4$ (0.02 g l^{-1}), KH_2PO_4 (2.8 g l^{-1}), KCl

(1.6 g l^{-1}), NH_4Cl (1.0 g l^{-1}), urea (25.0 g l^{-1}), creatinine (1.1 g l^{-1}) and dextrose 0.3%.

Silicone

Coupons (1 × 1 cm^2) were cut from a 1 mm layer of silicone (Neves & Neves, Trofa, Portugal). This material was chosen due to its similarity to the material used in indwelling devices, including urinary catheters. All coupons were cleaned by immersion in ultrapure water, followed by immersion in 50% (v/v) ethanol for 4 h. After rinsing with ultrapure water and air-driyng, the coupons were autoclaved for 15 min at 121°C.

Candida surface properties

Contact angle measurement

Hydrophobicity was evaluated through contact angle measurements and using the approach of van Oss *et al.* (1987, 1989). The measurements were made on *Candida* cell lawns deposited on membrane filters, after 2 h of equilibrium in AU pH 5.8, prepared according to Busscher *et al.* (1984). For this, suspensions of 5 ml of 10^8 cells ml^{-1} were filtered and deposited over solidified agar plates (2% agar and 10% glycerol) and dried at 37°C for 3–4 h to standardize the humidity level. Contact angles were measured by the sessile drop technique on the cell lawns using a contact angle measurement apparatus (model OCA 15 PLUS, DATAPHYSICS). The measurements were performed at room temperature, using three different liquids: water, formamide and 1-bromonaphtalene. Each assay was performed in triplicate and at least 20 contact angles per sample were measured.

Zeta potential measurement

The electric charge of the *Candida* strains under study was evaluated by zeta potential determination, after 2 h of equilibrium in AU pH 5.8 (1×10^7 cells ml^{-1}). Measurements were performed with a microelectrophoresis cell (DLS Nanosizer; Malvern). The applied voltage was 200 V, and each average value consisted of 25 recordings.

Adhesion assays

For the adhesion assays, silicone coupons were placed into 12-well microtiter plates (Orange Scientific) and 1 ml of the standardized cell suspension (1×10^7 cells ml^{-1} in AU) was added. For controls, silicone coupons were similarly processed but in the absence of *Candida* cells. The microtiter plates were incubated for 2 h at 37°C in an orbital shaker (120 rev/min). Following incubation, silicone coupons were placed into new 12-well culture plates and washed once with ultrapure water to remove non-adhered cells. The cells were then fixed with 1 ml of 100% (v/v) methanol, which was removed after 15 min of contact. The silicone coupons were then allowed to air dry at room temperature, and 1 ml of 1% (v/v) crystal violet (CV) was added and incubated for 5 min to stain the adhered cells. The silicone coupons were then gently washed twice with ultrapure water to remove the excess of CV and were allowed to dry at room temperature.

Coupons were visualized under light microscopy (Olympus BX51) coupled to a DP71 digital camera (Olympus) for acquisition of images (original magnification×400). At this magnification, each captured image was equivalent to 1.7×2.2 cm^2. For each surface analysed, at least 20 images were obtained across the entire silicone surface. The number of adhered cells per image was determined by image analysis using automated enumeration software (Sigma Scan Pro, Systat Software). The

results were then expressed as the number of cells per unit area of silicone (number cells cm^{-2}). All experiments were performed in triplicate and in three to five independent assays.

Biofilm formation assays

For biofilm formation assays, silicone coupons were placed in 12-well microtiter plates (Orange Scientific) and 1 ml of standardized cell suspension (1×10^7 cells ml^{-1} in AU) was added. The microtiter plates were incubated for 4, 8, 12, 24, 48 and 72 h at 37°C in an orbital shaker (120 rev/min). Every 24 h, 500 µl AU was removed and an equal volume of fresh AU added. For controls, silicone coupons were similarly processed but in the absence of *Candida*. Biofilm formation was assessed by total biomass quantification using CV staining (Stephanovic *et al.,* 2000; Silva *et al.,* 2009). Thus, after the defined times of incubation, the medium was aspirated and non-adherent cells removed by washing the silicone coupons with sterile water. The biofilm was then fixed with 1 ml of 100% (v/v) methanol, which was removed after 15 min of contact. The silicone coupons were allowed to dry at room temperature, and 1 ml of CV (1% v/v) was added to each well and incubated for 5 min. The silicone coupons were gently washed with sterile water and 1 ml of acetic acid (33% v/v) was added to release the CV from cells. The A_{570} of the obtained solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa). The final absorbance was standardized according to the area of silicone (absorbance cm^{-2}). Experiments were performed in triplicate and repeated in three to five independent assays.

Candida viability assays

The number of cultivable cells was determined by CFU enumeration. Briefly, after 4 and 72 h of biofilm formation (as described previously), silicone coupons were washed once in PBS (0.1M, pH 7) to remove loosely attached cells. Coupons of silicone were then immersed in 3 ml of PBS (0.1M, pH 7) in new 6-well tissue culture plates and sonicated (Ultrasonic Processor, Cole-Parmer) for 45 s at 30 W. Complete removal of adhered cells was confirmed by CV staining (as described previously). The obtained suspensions were vortexed vigorously for 5 min and then serial decimal dilutions (in PBS) were subcultured onto SDA and incubated for 24 h at 37ºC for c.f.u enumeration (log CFU cm^{-2}). Experiments were repeated in three occasions with individual samples evaluated in triplicate.

Confocal laser scanning microscopy (CLSM)

After 72 h of biofilm formation, silicone coupons were washed once with PBS (0.1M, pH 7), incubated for 40 min at 37°C in 1 ml of PBS containing the fluorescent stains FUN 1 (10 µM, Molecular Probes-Invitrogen) and concanavalin A Alexa Fluor 488 conjugate (25 µg ml^{-1} in PBS, Molecular Probes-Invitrogen) and observed by CLSM.

FUN1 (excitation wavelength=543 nm and emission=560 nm) is converted to orange-red cylindrical intravacuolar structures by metabolically active cells, while concanavalin A (excitation wavelength=488 and emission=505 nm) binds to glucose and mannose residues with the emission of green fluorescence. Stained silicone coupons were observed with a Leica TCS SP2 AOBS spectral confocal microscope.

Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) by applying Levene´s test of homogeneity of variance and the Tukey multiple-comparisons test, using SPSS software. All tests were performed with a confidence level of 95%.

Results

Hydrophobicity and zeta potential of non-*Candida albicans Candida* species

Candida cell surface physico-chemical characteristics are presented in Table IV.1.

Table IV.1 Water contact angle (Θ), values of the polar components of surface tension (γ^+, γ^-), degree of hydrophobicity (ΔG_{SWS}) and zeta potential (ζ) of non-*Candida albicans Candida* strains assayed

Species	Strain	θ (°) ± SD	γ^+ (mJm^{-2})	γ^- (mJm^{-2})	ΔG_{sws} (mJm−2)	ζ (mV) ± SD
C. parapsilosis	534638	19.4 ± 2.7	4.4	51.1	24.4	- 2.2 ± 0.0
	553877	17.3 ± 1.2	3.4	51.8	26.3	- 1.4 ± 0.0
	ATCC 22019	17.7 ± 2.5	3.6	51.6	25.8	-2.7 ± 0.5
C. tropicalis	519468	20.7 ± 1.3	3.7	49.0	23.2	-2.6 ± 0.1
	544123	24.0 ± 1.3	8.4*	47.2*	15.5*	-2.6 ± 0.6
	ATCC 750	20.5 ± 2.3	4.5	49.8	23.0	-3.8 ± 0.7
C. glabrata	562123	17.4 ± 2.4	3.4	50.6	25.1	-4.1 ± 0.6$^{\Delta}$
	513100	19.2 ± 2.4	4.9	51.3	23.7	- 4.1 ± 0.9$^{\Delta}$
	ATCC 2001	19.6 ± 2.2	3.1	53.0	28.4	-4.2 ± 0.6$^{\Delta}$

* Statistically different from the other strains (P<0.05).
† Strains that are statistically similar (P>0.05) but different from (P<0.05) those of other species.

The hydrophobicity of the yeast cell surfaces (ΔG_{sws}) expresses the free energy of interaction between two identical surfaces (s) immersed in water (w), as proposed by van Oss *et al.* 1995. Water contact angles were

used as a qualitative indication of cell surface hydrophobicity, with values below 65° indicating a hydrophilic surface (Vogler, 1998).

Similar water contact angle values were obtained for all NCAC strains, ranging from 17.3° ± 1.2 (*C. parapsilosis* 553877) to 24.0° ± 1.3 (*C. tropicalis* 544123), indicating that the different strains were of similar cell surface hydrophobicity ($P>0.05$) which were positive therefore indicating hydrophilic surfaces. Regarding surface tension components, all strains showed higher values of the electron donor parameter (γ-) comparing to the electron acceptor parameter (γ+). *Candida tropicalis* 544123 showed, again, the highest value of electron acceptance while *C. glabrata* ATCC 2001 revealed the lowest value of the electron donor parameter.

Table IV.1 summarizes the zeta potential values obtained for different NCAC strains when immersed in AU pH 5.8. All strains had a negative zeta potential value, ranging from -4.2 ± 0.6 mV to -1.4 ± 0.0 mV. Furthermore, *C. glabrata* strains presented the most negative values, which were statistically lower than *C. parapsilosis* and *C. tropicalis* strains ($P<0.05$).

Adhesion of non-*Candida albicans Candida* species to silicone

Figure IV.1 presents the results of the extent of adhesion of NCAC species (*i.e*. *C. glabrata*, *C. tropicalis* and *C. parapsilosis*) to silicone in the presence of AU. It was evident that all NCAC strains adhered to silicone, although differences were observed according to species and strains. Strain variation was particularly evident for *C. parapsilosis*, with *C. parapsilosis* 534638 displaying the highest number of cells adhered to silicone ($2.2 \times 10^6 \pm 7.5 \times 10^5$ cells cm^{-2}).

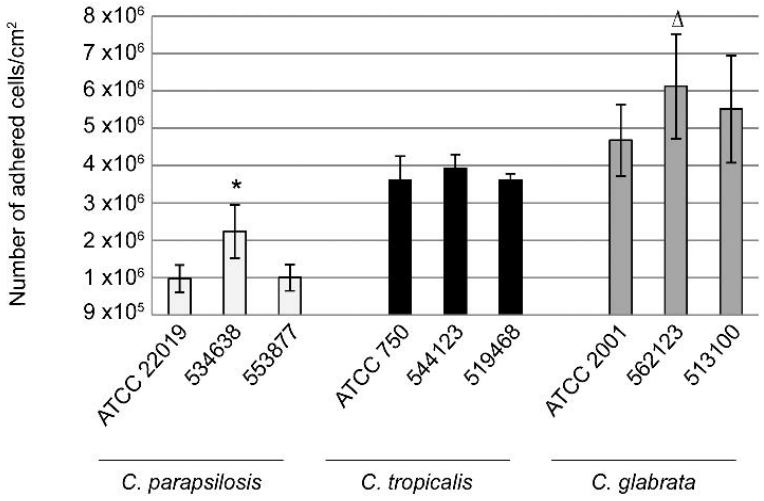

Figure IV.1 Number of cells of non-*candida albicans Candida* species per unit area adhered to silicone after 2 h in the presence of AU. Error bars represent standard deviation. *Statistically different from the other strains of the same species (P<0.05); $^{\Delta}$statistically different from all strains of other species (P<0.05).

This was significantly higher than *C. parapsilosis* 553877 (P=0.024) and the reference strain *C. parapsilosis* ATCC 22019 (P=0.023). Compared with *C. tropicalis* and *C. glabrata*, *C. parapsilosis* adhered to the silicone coupons at a significantly lower (P<0.05) level. Strains of *C. glabrata* adhered at equivalent levels to silicone (P>0.05), with the highest number of adhered cells ($6.2x10^6$ cells cm^{-2}) evident for *C. glabrata* 562123. *Candida tropicalis* strains equally showed similar adhesion levels with little inter-strain difference (P>0.05).

Biofilm formation of non-*Candida albicans Candida* species on silicone

Biofilm quantification was performed through total biomass quantification by CV staining. Figure IV.2 shows the analysis of biofilm development (4, 8, 12, 24, 48 and 72 h of incubation) for *C. parapsilosis*, *C. tropicalis* and *C. glabrata* strains, on silicone in the presence of AU.

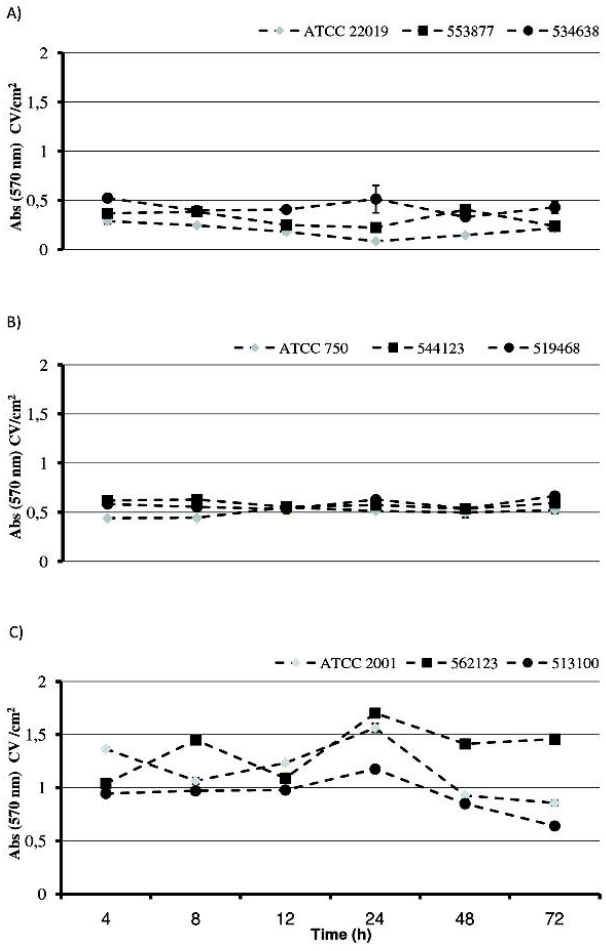

Figure IV.2 Abs (570 nm) of CV solutions obtained from biofilms of non-*Candida albicans Candida* species formed on silicone in the presence of AU. **(A)** *C. parapsilosis;* **(B)** *C. tropicalis;* and **(C)** *C. glabrata* strains. Error bars represent standard deviation.

It was evident that biofilms of *C. glabrata* had a higher total biomass than those of *C. parapsilosis* and *C. tropicalis* at all measured time points (P<0.05). *Candida tropicalis* and *C. parapsilosis* strains produced biofilm biomass of similar levels (P>0.05), whilst *C. glabrata* strains exhibited a more heterogeneous behaviour under the test conditions. No statistical differences were observed between biomass values obtained at 4 and 72 h in the case of *C. tropicalis* and *C. parapsilosis* strains (P>0.05). In contrast, biomass values for *C. glabrata* 513100 and ATCC 2001 strains decreased significantly after 48 h of incubation (P<0.05).

Silicone coupons incubated for 72 h were also examined by fluorescence CLSM (Figure IV.3).

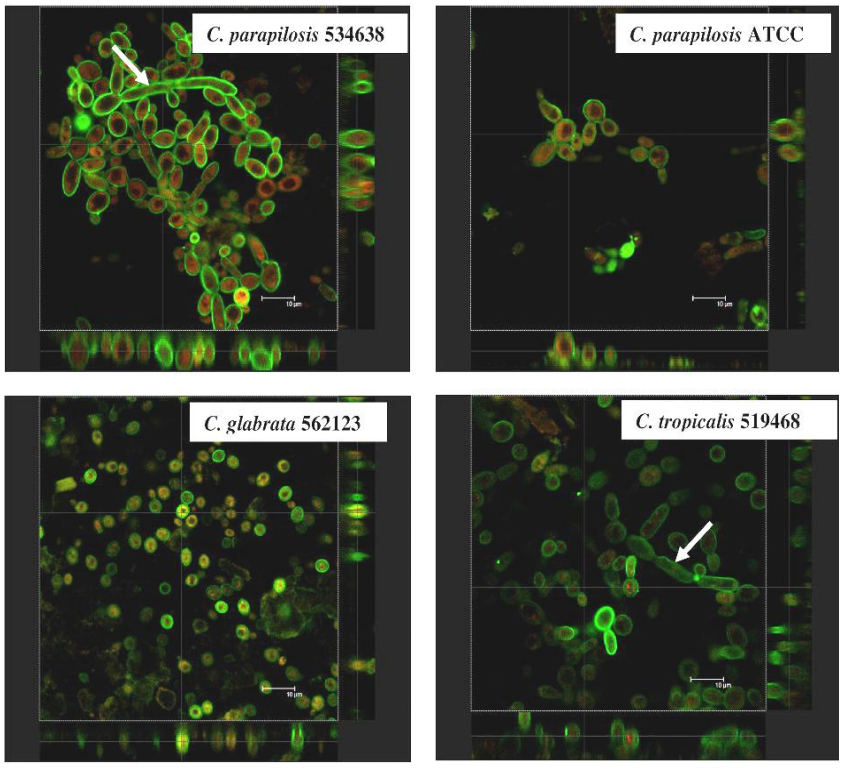

Figure IV.3 CLSM images of non-*Candida albicans Candida* species 72 h growth on a silicone surface in the presence of urine. Arrows indicate the presence of hyphal morphologies. Magnification × 600.

The intense green fluorescence, resulting from concanavalin A binding to polysaccharides, outlines the live and dead cell wall of yeasts, while the red/orange colour, due to FUN 1 staining, identifies active cells. Thus, confocal images showed an absence of extracellular polymeric material (green coloration) at the last period of incubation (cells separated by regions lacking fluorescence), though this is not surprising, since the concentration of polymers within the matrix is typically low. The observed area of red/orange fluorescence, confirmed the presence of higher amounts of metabolically active cells after 72 h adhesion on silicone in the presence of urine. Furthermore, *C. parapsilosis* and *C. tropicalis* strains presented filamentous morphologies which were not evident with *C. glabrata* strains.

Discussion

UTI is the most common type of nosocomial infection and is frequently associated with the use of indwelling urinary catheters (Febré *et al.,* 1999). *Candida* species are responsible for around 15% of UTIs (Amer *et al.,* 2004) and there is an increasingly recognised involvement of NCAC species (Harris *et al.,* 1999; Kauffman *et al.,* 2000; Krcmery *et al.,* 2002; Ruan *et al.,* 2008). Silicone rubber is the biomaterial frequently used to manufacture urinary catheters, and given the involvement of NCAC in UTIs, this present study aimed to assess the ability of NCAC species to colonize silicone in the presence of AU.

All NCAC species (*i.e. C. glabrata*, *C. parapsilosis* and *C. tropicalis*), were able to adhere to silicone (Figure IV.1), which is in agreement with other studies examining *Candida* species adherence to silicone rubber (Busscher *et al.,* 1997; Tamura *et al.,* 2003). However, it is important to note that *C. glabrata* strains adhered to a higher extent compared with *C. parapsilosis* and *C. tropicalis*. In fact, *C. glabrata* strains have frequently been isolated from patients with candiduria (Harris *et al.,* 1999; Manzano-Gayosso *et al.,* 2008) and recognized as potentially pathogenic in hospitalized and catheterized patients (Fidel *et al.,* 1999). As previously reported (Trofa *et al.,* 2008), *C. parapsilosis* is not a frequent etiological agent of UTIs. This was also evident in a recent epidemiological study of 100 candiduria cases (between 1999 and 2004) in a paediatric hospital in Brazil. In this study, *C. parapsilosis* was only isolated on four occasions compared with 56 for *C. albicans*, 20 for *C. tropicalis* and 11 for *C. glabrata* (da Silva *et al.,* 2007). Furthermore, despite the apparently reduced ability to adhere to silicone, *C. parapsilosis* strains exhibited a higher degree of intra-species heterogeneity compared with the two other species examined (Figure VI.1). This intra-species variation by *C. parapsilosis* in its

adherence to different biomaterials has been reported previously (Cassone *et al.,* 1995; Panagoda *et al.,* 2001; Trofa *et al.,* 2008). Such findings undoubtedly reflect inherent physiological differences between species and strains and could have significance with respect to pathogenic potential.

It is well known that cell surface properties, such as hydrophobicity and zeta potential, play an important role in adhesion of microorganisms to the surface of medical devices (Cai *et al.,* 1994; Anil *et al.,* 2001; Henriques *et al.,* 2002). As a result, we investigated the relationship between adhesion and hydrophobicity and zeta potential parameters. The results showed that the water contact angle values obtained for the different *Candida* species were very similar, which differs from results previously reported by Anil *et al.* (2001), who demonstrated that hydrophobic behaviour varied amongst different *C. albicans* strains, although grown in different conditions. Furthermore, this work showed that *C. parapsilosis*, *C. tropicalis* and *C. glabrata* strains were all hydrophilic and this fact does not affect the adherence abilities. In addition, all the tested strains analysed had similar contact water angle values and this was despite *C. glabrata* exhibiting significantly higher adherence to silicone. Camacho *et al.* (2007) also did not find a correlation between hydrophobicity and adherence for *Candida* species on siliconized latex urinary catheters, demonstrating that cell hydrophobicity alone was not a predictor for the adhesion phenomenon. Since *Candida* cells and silicone (Sousa *et al.,* 2009) are negatively charged, it was expected that adhesion of strains with greater negative zeta potentials would be hindered by electrostatic repulsion. Interestingly, the highest number of adhered cells was found for *C. glabrata* strains, which actually had the higher negative values of zeta potential, thus, highlighting that zeta potential is also not primarily responsible for adherence potential. Additionally, according to

several authors (Verran *et al.,* 1997; Carlen *et al.,* 2001), micro-organism adhesion is dependent on the surface roughness of the substratum. Depending on the extent of surface roughness, areas suitable for the physical retention of microorganisms to aid colonization are present. Sousa *et al.,* (2009) recently demonstrated that silicone exhibits large numbers of depressions and grooves, and since it is known that *C. glabrata* cells are generally smaller (1-4 µm) than *C. tropicalis* (4-8 µm) and *C. parapsilosis* (2.5-4 µm) (Odds, 1998) it is tempting to speculate that the silicone rubber coupons used in this study had a surface roughness optimal for *C. glabrata* retention.

Initial attachment of individual cells to a substratum is closely followed by cell division, proliferation, and biofilm development (Ramage *et al.,* 2006). Under the conditions used in these experiments and despite extensive adherence of NCAC species, biofilm formation was not subsequently detected (Figure IV.2). Nevertheless, it was possible to observe differences among the NCAC species under study regarding total biomass. As found in the adhesion assays, *C. glabrata* colonization yielded a higher total biomass than that of *C. parapsilosis* and *C. tropicalis*. It is known that mature *Candida* biofilms are organised into structured communities embedded within a matrix of extracellular material that is produced by the biofilm cells (Al-Fattani *et al.,* 2006; Silva *et al.,* 2009). Formation of mature biofilms and consequent production of matrix is strongly dependent on environmental conditions, such as medium composition, pH, oxygen, species and strains (Jain *et al.,* 2007; Ramage *et al.,* 2006). In fact, these particular isolates are not capable of forming dense 3D biofilms and produce enough matrix (Figure IV.3). However, it should be emphasized that only few isolates were used and it other isolates belonging to same species may be able to form stronger biofilms. Furthermore, the authors verified that, as previously recognized (Jain *et al.,*

2007; Uppluri *et al.,* 2009), an increase of sugars (which could be representative of diabetic patients), is a requirement for increased biofilm formation (data not shown). Furthermore, fungal infections are also more common in patients with diabetes, particularly those involving *Candida* species, occasionally causing dramatic clinical repercussions with obstruction of the urinary catheter (Patterson *et al.,* 1995). However, it is important to stress that the composition of AU was formulated to mimic the natural urine of non-diabetic patients (pH 5.6 with a residual quantity of sugar).

Enumeration of CFU and confocal observation revealed that whilst NCAC species were unable to form dense biofilms on the silicone in the presence of AU (Figure IV.3), *Candida* cells remained viable over the 72 h period of study [mean log CFU (cm biofilm)$^{-2}$: 5.3 for *C. parapsilosis*, 5.4 for *C. tropicalis* and 6.1 for *C. glabrata*). This is highly important as it implies that under appropriate conditions *Candida* can proliferate within the catheter environment and potentially infect vital organs in the host (such as bladder and kidneys).

In summary, this work demonstrates that the tested NCAC species were able to colonize and survive on the silicone surface in the presence of AU. Additionally, it was shown that *C. glabrata* strains exhibited higher colonization of silicone surfaces compared with *C. tropicalis* and *C. parapsilosis*. This finding is potentially significant, not just for UTI in catheterized patients, but also for other patients who have inserted silicone medical devices including those patients who receive transplants or who are admitted into intensive care units. Importantly, such patients would appear to have a higher incidence of *C. glabrata* colonization compared with other NCAC species (Guglielmo *et al.,* 1994; Krcmery, 1997; Manzano-Goyasso *et al.,* 2008).

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References

Amer FA, Mohtady HA, el-Behedy IM, *et al.,* (2004). Bacteria of nosocomial urinary tract infections at a university hospital in Egypt: identification and associated risk factors. *Infect Control Hosp Epidemiol* **25**: 895-897.

Anil S, Ellepol ANB, Samaranayake LP (2001). The impact of clorhexidine gluconate on the relative cell surface hydrophobicity of oral *Candida albicans*. *Oral Dis* **7:** 119-122.

Busscher HJ, Weerkamp AH, van der Mei HC, *et al.,* (1984). Measurements of the surface free energy of bacterial cell surface and its relevance for adhesion. *Appl Environ Microbiol* **48**: 980–983.

Busscher HJ, Geertsema-Doornbusch GI, van der Mei HC (1997). Adhesion to silicone rubber of yeasts and bacteria isolated from voice prostheses: influence of salivary conditioning films. *J Biomed Mater* **34**: 201-210.

Cai S, Simionato MR, Mayer MP, *et al.,* (1994). Effects of subinhibitory concentrations of chemical agents on hydrophobicity and in vitro adherence of *Streptococcus mutants* and *Streptococcus sanguis*. *Caries Res* **28**: 335-341.

Camacho DP, Gasparetto A, Svidzinski TIE (2007). The effect of chlorhexidine and gentian violet on the adherence of *Candida* spp. to urinary catheters. *Mycopathologia* **163**: 261-266.

Carlén A, Nikdel K, Wennerberg A, *et al.,* (2001). Surface characteristics and *in vitro* biofilm formation on glass ionomer and composite resin. *Biomaterials* **22**: 481-487.

Cassone A, De Bernardis A, Pontieri E, *et al.,* (1995). Biotype diversity of *Candida parapsilosis* and its relationship to the clinical source and experimental pathogenicity. *J Infect Dis* **171**: 967-975.

Chandra J, Mukherjee PK, Leidich SD, *et al.,* (2001a). Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. *J Dent Res* **80**: 903-908.

Chandra J, Kuhn DM, Mukherjee PK, *et al.,* (2001b) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* **183**: 5385-5394.

Chow JK, Golan Y, Ruthazer R, *et al.,* (2008). Factors associated with candidemia caused by non-*albicans Candida* species versus *Candida albicans* in the intensive care unit. *Clin Infect Dis* **46**: 1206-1213.

Costerton JW, Lewandowski Z, Caldwell DE, *et al.,* (1995). Microbial biofilms. *Annu Rev Microbial* **49**: 711-745.

Crump JA, Collignon PJ (2000). Intravascular catheter-associated infections. *Eur J Clin Microbiol Infect Dis* **19**: 1-8.

da Silva EH, Ruiz S, Matsumoto FE, *et al.,* (2007). Candiduria in a public hospital Sao Paulo (1999-2004): characteristics of the yeast isolates. *Rev Inst Med Trop S Paulo* **49**: 349-353.

Douglas LJ (2002). Medical importance of biofilms in *Candida* infections. *Rev Iberoam Micol* **19**: 139-143.

Douglas LJ (2003). *Candida* biofilms and their role in infection. *Trends Microbiol* **11**: 30-36.

Febré N, Silva V, Medeiros EAS, *et al.,* (1999). Microbiological characteristics of yeasts isolated from urinary tracts of intensive care unit patients undergoing urinary catheterization. *J Clin Microbiol* **37**:1584-1586.

Fidel PL, Vasquez JA, Sobel J (1999). *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin Microbiol Rev* **12**: 80-96.

Guglielmo BJ, Stoller ML, Jacobs RA (1994). Management of Candiduria. *In J Antimicrob Agents* **4**: 135-139.

Harris AD, Castro J, Sheppard DC, *et al.,* (1999). Risk factors for nosocomial candiduria due *Candida glabrata* and *Candida albicans*. *Clin Infect Dis* **29**: 926-928.

Henriques M, Gasparetto K, Azeredo J, *et al.,* (2002). Experimental methodology to quantify *Candida albicans* cell surface hydrophobicity. *Biotechnol lett* **24**: 1111-1115.

Jain N, Kohli R, Cook GP, *et al.,* (2007). Biofilm formation by and antifungal susceptibility of *Candida* isolates from urine. *Appl Environ Microb* **73**: 1697-1703.

Odds FC. *Candida* and Candidosis, 2n edn. London, Bailliere Tindall, 1998.

Kauffman CA, Vazquez JA, Sobel JD, *et al.,* (2000). Prospective multicenter surveillance study of funguria in hospitalized patients. *Clin Infect Dis* **30**: 14-18.

Kojic EM, Darouiche RO (2004). *Candida* infections of medical devices. *Clin Microbiol Rev* **17**: 255-267.

Krcmery S. Urinary tract infections in renal transplant patients. In: Bergan T, editor. Urinary tract infections. Basel: Karger, **1997**: 48-52.

Krcmery V, Barnes AJ (2002). Non-*albicans Candida* spp. causing fungaemia pathogenicity and antifungal resistance. *J Hosp Infect* **50**: 243-260.

Manzano-Gayosso P, Hernández HF, Velásquez NZ, *et al.,* (2008). Candiduria in type 2 diabetes mellitus patients and its clinical significance. *Candida* spp antifungal susceptibility. *Rev Med Inst Mex Seguro Soc* **46**: 603-610.

Panagoda GJ, Ellepola ANB, Samaranayake LP (2001). Adhesion of *Candida parapsilosis* to epithelial and acrylic surfaces correlates with cell surface hydrophobicity. *Mycoses* **44**: 29-35.

Patterson JE, Andriole VT (1995). Bacterial urinary tract infections in diabetes. *Infect Dis Clin North Amer* **9**: 25-51.

Ramage G, Wickes BL, López-Ribot JL (2001). Biofilms of *Candida albicans* and their associated resistance to antifungal agents. *Am Clin Lab* **20**: 42-44.

Ramage G, Martínez JP, López-Ribot JL (2006). *Candida* biofilms on implanted biomaterials: a clinically significant problem*. FEMS Yeast Res* **6**: 979-986.

Richards MJ, Edwards JR, Culver DH, *et al.,* (1999). Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit Care Med* **27**: 887-892.

Ruan SY, Chen SY, Hsuek PR (2008). Persistent *Candida parapsilosis* funguria associated with an indwelling urinary tract stent for more than 7 years. *J Med Microbiol* **57**: 1585-1587.

Samaranayake YH, Jin Y, Yau JYY, *et al.,* (2005). *In vitro* method to study antifungal perfusion in *Candida* biofilms*. J Clin Microbiol* **43**: 818-825.

Schaberg DR, Culver DH, Gaynes RP. (1991). Major trends in the microbial etiology of nosocomial infection. *Am J Med* **91**: 72-75.

Scherer S, Stevens DA. (1987). Application of DNA typing methods to epidemiology and taxonomy of *Candida* species. *J Clin Microbiol* **25**: 675-679.

Shin JH, Kee SJ, Shin MG, *et al.,* (2002). Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. *J Clin Microbiol* **40**: 1244-1248.

Silva S, Henriques M, Martins A, *et al.,* (2009). Biofilms of non- *Candida albicans Candida* species: quantification, structure and matrix composition. *Med Mycol* **47**: 681-689.

Sousa C, Henriques M, Teixeira P, *et al.,* (2009). Influence of surface properties on the adhesion of *Staphylococcus epidermidis* to acrylic and silicone. *Int J Biomather* doi:10.1155/2009/718017

Sobel JD, Kauffman CA, McKinsey D, *et al.,* (2000). Candiduria: a randomized, double-blind study of treatment with fluconazole and placebo. *Clin Infect Dis* **30**: 19-24.

Stephanovic S, Vukovic D, Dakic I, *et al.,* (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Meth* 2000 **40:** 175-179.

Tamura NK, Gasparetto A, Svidzinski TIE (2003). Evaluation of the adherence of *Candida* species to urinary catheters*. Mycopathologia* **156**: 269-272.

Trofa D, Gacsér A, Nsanchuk J (2008). *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* **21**: 606-625.

van Oss CJ, Chaudhury MK, Good RJ (1987). Monopolar surfaces. *Adv Colloid Interface Sci* **28**: 35–64.

van Oss CJ, Ju L, Chaudhury MK, *et al.,* (1989). Estimation of the polar parameters of the surface tension of liquids by contact angle measurements on gels. *J Colloid Interface Sci* **128**:313–319.

Verran J, Maryan JC (1997). Retention of *Candida albicans* on acrylic resin and silicone of different surface topography. *J Prosthet Dent* **77**: 535-539.

Vogler EA (1998). Structure and reactivity of water at biomaterial surfaces. *Adv Colloid Interface Sci* **74**: 69-117.

Uppluri P, Dinakaran H, Thomas DP, *et al.,* (2009). Characteristics of *Candida albicans* biofilms grown in a synthetic urine medium. *J Clin Microbiol* **47**: 4078-4083.

Williams DW, Wilson MJ, Lewis MAO, *et al.,* (1995). Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol* **33**: 2476-2479.

Chapter V

Characterization of *Candida parapsilosis* infection of an *in vitro* reconstituted human oral epithelium

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Sónia Silva., Mariana Henriques, Rosário Oliveira, Joana Azeredo, Sladjana Malic, Samuel J Hooper, David W Williams. ***European Journal of Oral Sciences*** **(2009), 117**: 669-675.

Abstract

Oral candidosis is a common problem in immunocompromised patients, and whilst *C. albicans* is regarded as the principle cause of infection, other non-*Candida albicans Candida* (NCAC) species are increasingly being recognised as human pathogens. Relatively little is known about the virulence factors associated with NCAC species, and the aim of this study was to use a reconstituted human oral epithelium (RHOE) to examine epithelial infection of *C. parapsilosis*. Strains originating from the oral and vaginal mucosa and from the urinary tract were all shown to colonize RHOE in a strain-dependent manner. Strain differences were found in the colonizing morphology and in the extent of invasion of the RHOE. Low invasion of RHOE was detected for strains after 12 h, whereas extensive tissue damage was evident after 24 h when assessed using histological examination and lactate dehydrogenase activity determination. Tissue damage was reduced in the presence of pepstatin A, although *C. parapsilosis* invasion of the tissue was not inhibited. Real-time polymerase chain reaction of secreted aspartyl proteinase (*SAP)* genes (*SAPP1-3*) showed that expression was strain dependent, with an increased expression generally occurring for *Candida* infecting RHOE compared with planktonic equivalents. In summary, *C. parapsilosis* was not highly invasive of RHOE but did induce significant tissue damage which could relate to specific *SAP* gene expression.

Keywords: *Candida*; candidosis; *Candida parapsilosis*; oral epithelium

Introduction

Several species of *Candida* can colonize human mucosal surfaces as harmless commensals. Normally, infection (candidosis) does not occur because of efficient of the host by innate and adaptive immune responses. However, debilitation of the host often results in candidosis, which most frequently manifests as superficial infection of moist mucosal surfaces, such as the vagina and oral cavity. *Candida albicans* is regarded as the most prevalent species involved in both colonization and infection. However, in recent years an increased incidence of infection by non-*Candida albicans Candida* (NCAC) species has become evident (Samaranayake, 1990). This is significant because there are often inherent differences in susceptibility to traditional antifungals between *C. albicans* and NCAC species.

Candida parapsilosis is one such NCAC species that is now recognized as an important pathogen of debilitated individuals (Kcrmery *et al.,* 2000; Bonassoli *et al.,* 2005; Fridkin *et al.,* 2006). However, when compared with the extensively studied *C. albicans* (De Bernardis *et al.,* 1995; Schaller *et al.,* 1998; Bartie *et al.,* 2004; Jayatilake *et al.,* 2005; 2006; Malic *et al.,* 2007), relatively little is known about the associated virulence factors of *C. parapsilosis* (Jayatilake *et al.,* 2006; Gácser *et al.,* 2007). A greater awareness of the virulence determinants of this species are important, as not only would this provide an insight into pathogenic mechanisms but may also promote the development of more effective management strategies.

The virulence attributes of *Candida* species include the ability to adhere to host tissues, the ability to exhibit morphological alteration, and the ability to secrete hydrolytic enzymes, [*e.g.*, phospholipases and secreted aspartyl proteinases (Saps)] (Haynes, 2001; Lerman *et al.,* 2008;

Naglik *et al.,* 2008). The change in *Candida* species from being commensal orgamisms to becoming pathogenic and invasive has been related to altered expression of these virulence factors together with a debilitation in host defence mechanisms. Secreted aspartyl proteinase are considered to be potentially important enzymes that contribute to infection by promoting damage to the host mucosa, facilitating invasion of the organism into the epithelium (Zaugg *et al.,* 1992; Schaller *et al.,* 1998; Malic *et al.,* 2007). *Candida parapsilosis* possesses at least three genes encoding for Saps, designated *SAPP1* (Haynes, 2001), *SAPP2* (Monod *et al.,* 1994) and *SAPP3* (NCBI accession number AF339513).

The pathogenesis of mucosal candidosis has been investigated by several authors using animal models and primarily with *C. albicans* (Howlett, 1976; Zaugg *et al.,* 1992; De Benardis *et al.,* 1995; Samaranayake *et al.,* 2001; Naglik *et al.,* 2008). In recent years, a commercially available reconstituted human oral epithelium (RHOE) has successfully been used to investigate the *in vitro* mechanisms of tissue degradation caused by infection with *Candida albicans* (Schaller *et al.,* 1998, 1999, 2002; Bartie *et al.,* 2004; Jayatilake *et al.,* 2005, 2006; Malic *et al.,* 2007).

The aim of the present study was to use the RHOE model, coupled with confocal laser scanning microscopy (CLSM) and real-time polymerase chain reacyion (PCR), to investigate epithelial infection with, and virulence of, *C. parapsilosis*. Specifically, a comparison of strains originating from different body sites was made in terms of tissue colonization, invasion and *SAP* gene expression.

Materials and methods

Organisms

A total of six clinical isolates of *C. parapsilosis,* originally recovered from the oral cavity (strains AD and AM2), vagina (strains 491861 and 513143), and urinary tract (strains 534638 and 553877) were used in this study. A reference strain of *C. parapsilosis* from the American Type Culture Collection (ATCC 22019^T, Manassas, VA, USA) was also examined. Oral isolates were obtained from the Biofilm Group of the Centre of Biological Engineering, Minho University (Braga, Portugal) and were originally obtained from the Clinic of Dentistry, Congregados (Braga, Portugal). Isolates from the vaginal and urinary tract were obtained from the Hospital of S. Marcos collection, (Braga, Portugal). The identity of all isolates was initially confirmed by PCR-based sequencing using specific primers (ITS1 and ITS4) that are specific for the 5.8S subunit gene (Williams *et al.,* 1995).

Isolate preparation

For each experiment, isolates were subcultured on sabouraud dextrose agar (Merck, Darmstadt, Germany) for 48 h at 37°C. The isolates were then cultured in Yeast Nitrogen Base medium (BD Diagnostics, Cowley, UK) supplemented with 0.5 % (w/v) glucose, for 12 h at 37°C. After incubation, the yeast cells were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS; pH 7). The yeasts were then enumerated using an Improved Neubauer haemocytometer and adjusted to a concentration of 2×10^6 cells ml^{-1}.

In vitro reconstituted human oral epihelium (RHOE) infection model

Commercially available human oral epithelium (human keratinocytes derived from a squamous carcinoma of the buccal mucosa, cell line TR 146; Skinethic Laboratory, Nice, France) was placed in MCDB 153-defined medium (Clonetics, San Diego, USA) containing 5 µg ml^{-1} insulin, 1.5 mM $CaCl_2$, 25 µl ml^{-1} gentamicin, and 0.4 µg ml^{-1} hydrocortisone, in 24-well tissue culture plates according to the manufacturer's instructions. Standardized suspensions of *C. parapsilosis* (1 ml of MCDB 153 defined medium containing 2×10^6 cells in) were subsequently placed directly onto 0.5 cm^2 RHOE tissue inserts. Control samples were inoculated with 1 ml of medium devoid of *C. parapsilosis*. Infected tissues were incubated at 37°C in a 5% CO_2 environment with saturated humidity for 12 h or 24 h. In addition, planktonic cultures of *C. parapsilosis* were prepared using the same tissue culture medium and incubation conditions, but without the RHOE. These planktonic cultures were subsequently used for standardizing lactate dehydrogenase (LDH) activity in experiments assessing tissue damage and also as planktonic controls for *SAP* gene-expression studies.

After incubation, the tissues were rinsed twice in PBS to remove non-adherent *C. parapsilosis* and then bisected, with one half of the tissue used for CLSM analysis and the other for real time-PCR studies. The culture medium was also analyzed for *Candida*-induced damage using a LDH activity assay. In addition, in two separate experiments, the Sap inhibiter, pepstatin A (Sigma, St Louis, MO, USA), was added to selected tissue inserts to assess the effect of Saps on the infection process. Briefly, pepstatin A was dissolved in 100% methanol and added directly to the prepared *Candida* inoculum to give a final pepstatin A concentration of 15

µg ml^{-1}. These tissue inserts were analyzed (24 h after infection) to determine invasion and evaluate the production LDH.

Confocal laser scanning microscopy (CLSM)

The degree of colonization and morphological characteristics of *C. parapsilosis* on the surface of fresh tissues was assessed by CLSM following direct labelling of the *Candida* with 100 µl of concanavalin A lectin conjugated with Alexa 594 (Molecular Probes-Invitrogen, Paisley, UK: 25 µg ml^{-1} in PBS) for 20 min at room temperature. Briefly, infected tissue was fixed in 2% (v/v) paraformaldehyde (in ultra-pure water) for 24 h at 4°C and embedded in paraffin wax using standard histological techniques. Tissues sections (20 µm) were cut and placed onto Histobond+ -coated microscope slides (Raymond A. Lamb, East Sussex, UK), de-waxed, and processed through xylene, ethanol, and water before staining. To visualize infection by *C. parapsilosis*, prepared sections were directly labelled with 50 µl Alexa 594-conjugated concanavalin A lectin (25 µg ml^{-1} in PBS) for 20 min at room temperature. For cell nucleus detection, keratinocytes within the RHOE sections were stained with Hoechst 33258 (Sigma-Aldrich; St Louis, MO, USA; 1 µg ml^{-1}) for 30 min at room temperature before washing in PBS. Tissues were then mounted using Vectashield (Vector Laboratories, Peterborough, UK) fade-retarding mountant. Stained sections were viewed and analyzed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany).

Lactate dehydrogenase (LDH) assay

The release of LDH from tissues into the medium was used as a measurement of cell damage. The LDH concentration in the medium from

control and infected tissues was measured at 24 h using the CytoTox-ONE kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The LDH activity of *Candida* cells in cell culture medium devoid of tissue was measured and subtracted from the LDH activity of the tissue infected with yeast. The LDH released during infection with the different *C. parapsilosis* strains was then expressed relative to the untreated control tissue. All experiments were performed in duplicate.

Analysis of *SAP* gene expression

Tissues for RNA extraction were stored at 4°C in 2-ml microtubes containing RNAlater (Ambion, Huntington, UK) solution. Before RNA extraction, lysis buffer was prepared by adding 10 µl of *ß*-mercaptoethanol per ml of RLT buffer (Qiagen, Crawley, UK). Then, 600 µl of lysis buffer and glass beads (0.5 mm diameter, approximately 500 µl) were added to each tissue and this mix was homogenized twice for 30 s using a Mini-Bead-Beater-8 (Stratech Scientific, Soham, UK). After disruption of tissue, the RNeasy Mini Kit (Qiagen) was used to complete total RNA extraction according to the manufacturer's recommended protocol. Potential DNA contamination was removed by RNase-Free DNase I (Qiagen) treatment. RNA was also extracted, using the same approach, from *C. parapsilosis* planktonic cells grown in MCDB 153-defined medium at 37°C in a 5% CO_2 environment for 24 h.

Primers for real time-PCR were designed using Primer3 web-based software (http://fokker.wi.mit.edu\cgi-bin\primer3\primer3_www.cgi) and are listed in Table V.1. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from *C. parapsilosis* ATCC 22019^T genomic DNA. Control *S14* primers were also used (Cairns *et al.,* 1997) to detect human recombinant DNA.

Table V.1 Primers used for real time-PCR analysis of *SAP* and control gene expression

Sequence (5'→ 3')	Primer	Target	PCR product size (bp)
AGTGGTCGTCAAACCACTCC	Forward	*SAPP1*	219
GACGGAAGCAAGCGAAATAG	Reverse		
TTACTTGCCTGACAGCATCG	Forward	*SAPP2*	277
CGCATAAGCGTGTCTCAAAA	Reverse		
GCTCAAGGTGCTGCTATTCC	Forward	*SAPP3*	253
TTGCATCAATGACCCAGAAA	Reverse		
ATGATAGAGTTGAAAGTAGTTTGGTCAATA	Forward	*ACT1*	350
ACTACTGCTGAAAGAGAAATTGTTAGAGAC	Reverse		
CAGGTCCAGGGGTCTTGGTCC	Forward	*S14*	143
GGCAGACCGAGATGAATCCTCA	Reverse		

From each sample, 10 µl of extracted RNA was used for complementary DNA (cDNA) synthesis. Firstly of all, the RNA was incubated with 5µl (50 µg ml^{-1}) of oligo-dT primer (Promega, Southampton, UK) at 70°C for 5 min. Then, this preparation was added to a reaction mixture containing RT Buffer [50 mM Tris (pH 8.3), 3 mM $MgCl_2$, 10 U of RNasin Plus RNase inhibitor (Promega)], 20 µM of each deoxynucleoside triphosphate (dNTP) and 200 U of Moloney Murine Leukaemia Virus-Reverse Transcriptase (MMLV-RT, Promega), in a final reaction volume of 50 µl. Synthesis of cDNA was then performed at 42°C for 1 h. The reaction was stopped by heating at 95°C for 5 min.

Real-time PCR was used to determine the relative levels of *SAPP1–3* mRNA transcripts in the RNA samples, with *ACT1* used as a reference candidal housekeeping gene (Rossignol *et al.,* 2007). A 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) was used according to the manufacturer's instructions. Each reaction mixture consisted of the

working concentration of Power SYBR® Green PCR master mix (Applied Biosystems), 300 nM forward and reverse primer, and 1 µl of cDNA, in a final reaction volume of 20 µl. Negative (water) controls were included in each run. The relative quantification of *SAPP1-3* gene expression was performed by the ΔC_T (threshold cycle) method, using the control gene (*ACT1*) to normalize the data. Each reaction was performed in triplicate and mean values of relative expression analyzed for each *SAP* gene.

Results

In vitro RHOE infection

The surface colonization and morphology of fresh RHOE tissues was examined after 24 h incubation with *C. parapsilosis* (Figure V.1 and Table V.2). The results showed that all *C. parapsilosis* strains were able to colonize the RHOE surface, although the extent of colonization was strain dependent. *Candida parapsilosis* AD (oral strain) and *C. parapsilosis* 534638 (urinary tract strain) yielded the highest level of colonization of the epithelial surface, whilst strain AM2 (oral) colonized the tissue to a lower extent (Figure V.1).

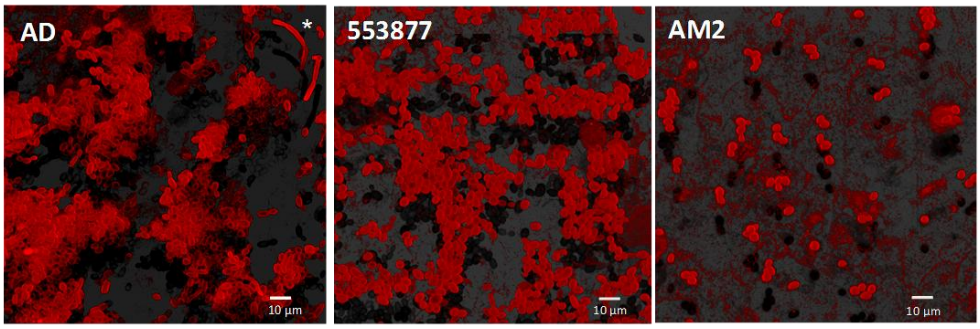

Figure V.1 Surface colonization of *Candida parapsilosis* strains infecting reconstituted human oral epithelium after 24 h of incubation: **(A)** *C. parapsilosis* AD showing extensive colonization (+++), **(B)** *C. parapsilosis* 553887 showing moderate colonization (++) and **(C)** *C. parapsilosis* AM2 demonstrating sparse colonization (+). Presence of pseudohyphal form **(*)**.

Table V.2 *Candida parapsilosis* infection profile of reconstituted human oral epithelium (at 12 and 24 h), as assessed using confocal laser scanning microscopy

***C. parapsilosis* strain**	**Origin**	**Surface colonization**	**Strain morphology in presence of tissue**	**Invasion/integrity of RHOE**	
				12 h	**24 h**
AD	**Oral**	**+++**	Yeast and pseudohyphae	Moderate/undamaged	Moderate/partial damage
AM2		**+**	Yeast	None/undamaged	None/undamaged
534638	**Urinary**	**++**	Yeast and pseudohyphae	Moderate/undamaged	Moderate/partial damage
553877		**++**	Yeast	Low/undamaged	Moderate/damaged
491861	**Vaginal**	**+++**	Yeast and pseudohyphae	-	Moderate/partial damage
513143		**++**	Yeast	Moderate/undamaged	None/partial damage
ATCC 22019	**Reference**	**++**	Yeast and pseudohyphae	-	Moderate/damage

Moreover, the different *C. parapsilosis* strains presented diverse cell morphologies on the RHOE surface. *Candida parapsilosis* AD (oral strain), *C. parapsilosis* 534638 (urinary tract strain), *C. parapsilosis* 491861 (vaginal strain) and *C. parapsilosis* ATCC 22019^{T} formed a network of yeasts and pseudohyphae, while the other strains appeared devoid of pseudohyphae (Figure V.1 and Table V.2).

To assess the invasive capability of *C. parapsilosis*, RHOE tissues were fixed, sectioned, stained, and analyzed using CLSM, 12 h and/or 24 h after incubation (Figures V.2, V.3; Table V.2).

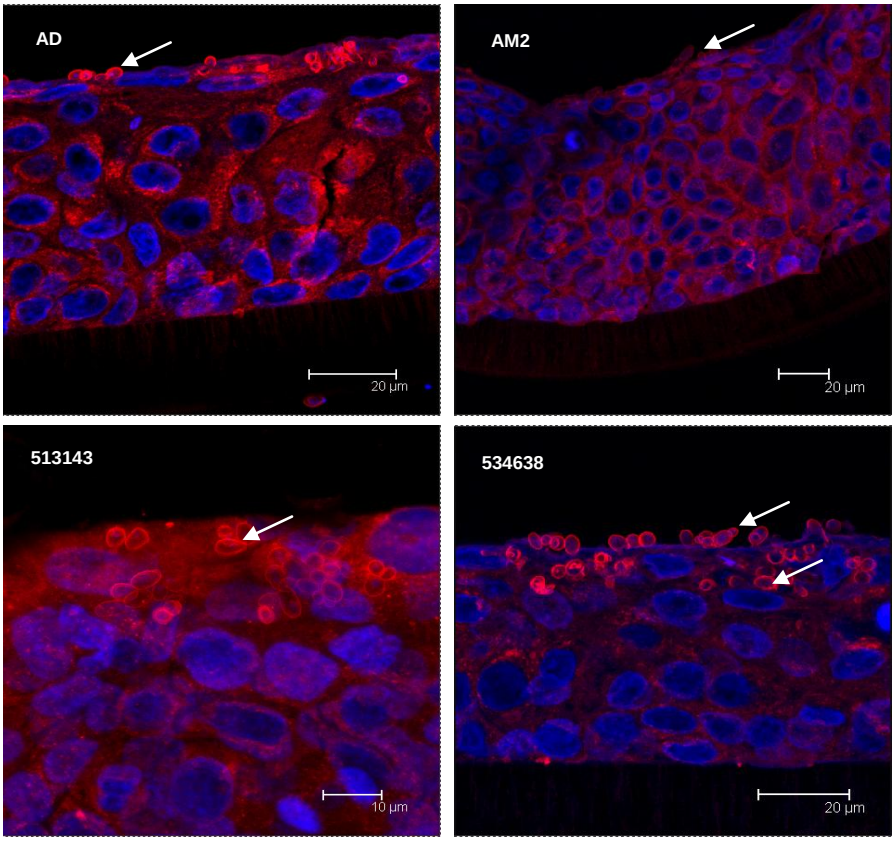

Figure V.2 Confocal laser scanning microscopy of *Candida parapsilosis* strains infecting human oral epithelium after 12 h infection. Arrows indicate yeast cells.

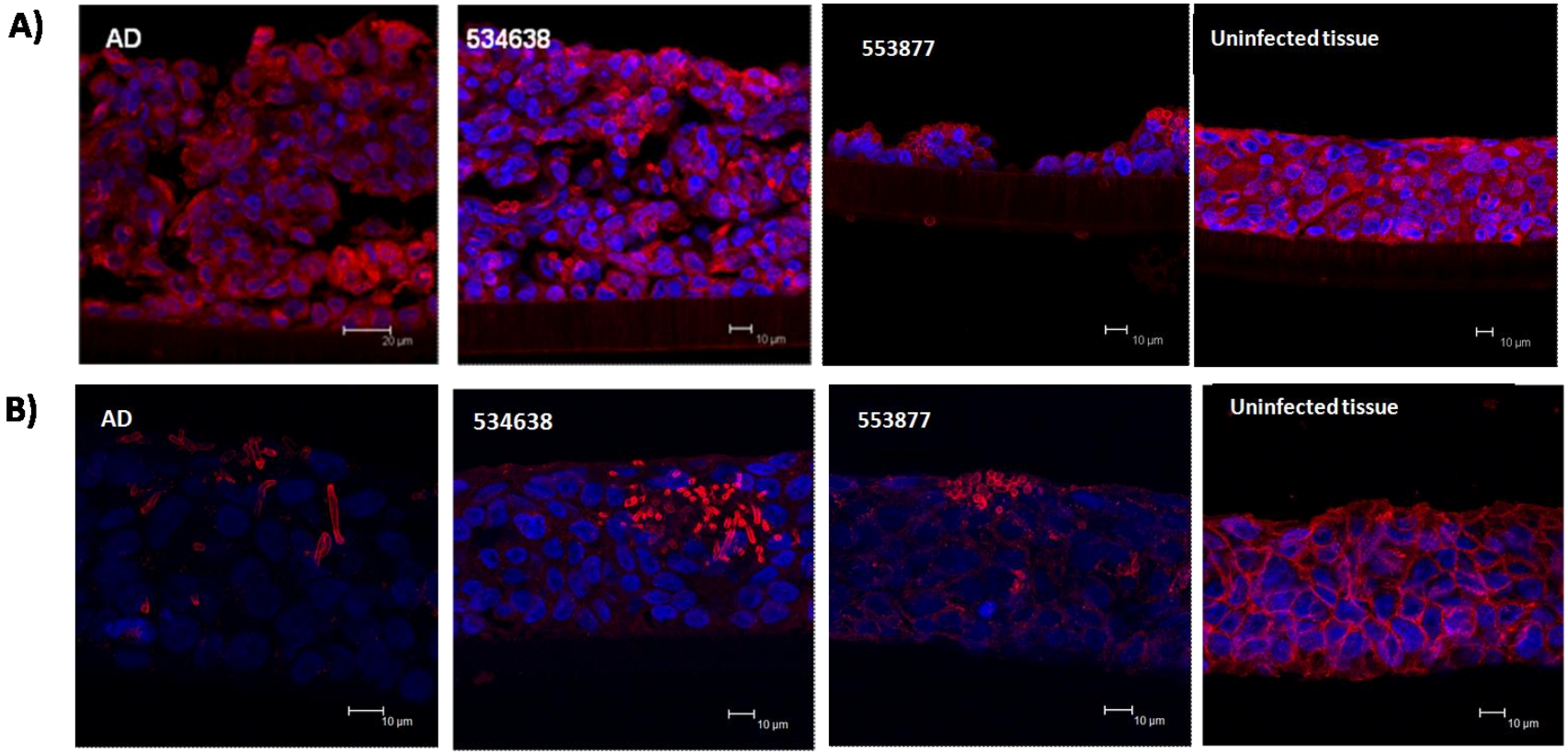

Figure V.3 Confocal laser scanning microscopy of *Candida parapsilosis* infecting human oral epithelium after 24 h of incubation in the absence **(A)** and presence **(B)** of pepstatin A.

Figure V.2 illustrates the ability of *C. parapsilosis* to invade the RHOE, but not through all the epithelial layers. In fact, after 12 h incubation, it was only possible to detect clusters of cells within the superficial keratinocyte layers, never reaching deeper than the second or third cell layer (Figure V.2).

After 24 h incubation (Figure V.3 and Table V.2) the majority of strains under study caused damage to the tissue structure.

Infection with *C. parapsilosis* 553877 (urinary tract strain) and ATCC 22019^T resulted in detachment of the keratinocyte layers, leading to a loss of tissue integrity. Infection with *C. parapsilosis* AD (oral strain) and *C. parapsilosis* 534638 (urinary tract strain) induced vacuolization between keratinocytes, resulting in a high level of disorganization of the epithelium structure. By contrast, changes caused by *C. parapsilosis* 513143 and *C. parapsilosis* 491861 (both vaginal strains) were not as extensive, but still resulted in structural damage of the epithelium. Tissue alteration caused by *C. parapsilosis* AM2 (oral strain) was limited, with few yeast found to attach to the oral epithelium and the tissue structure remaining similar to that of the uninfected control.

To quantify tissue damage, the levels of LDH released were determined after 24 h after infection with *C. parapsilosis* (Figure V.4). Co-culture of tissue with each of the isolates caused a marked increase in the LDH levels compared with uninfected controls. The LDH results showed a positive correlation with the extent of tissue damage seen microscopically (Figure V.3A and Table V.2). *Candida parapsilosis* AD and *C. parapsilosis* ATCC 22019^T generated the highest levels of LDH and tissue damage, whereas *C. parapsilosis* AM2 and *C. parapsilosis* 513153 produced relatively lower levels of LDH and tissue damage.

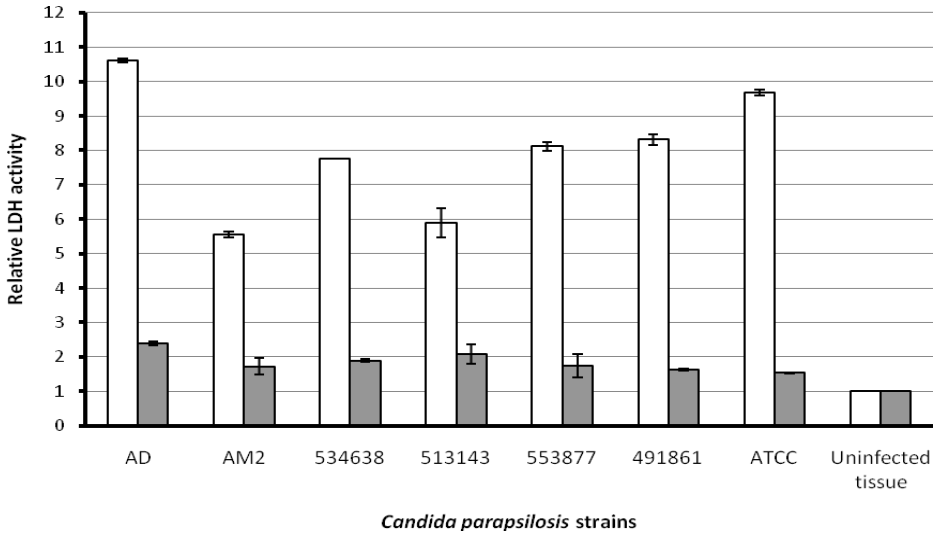

Figure V.4 Relative lactate dehydrogenase (LDH) activity measured in the human oral epithelium tissue culture supernatant after 24 h incubation with different *Candida parapsilosis* strains in absence (□) and in presence (■) of pepstatin A.

In order to help understand how *C. parapsilosis* induced RHOE damage, the effect of the specific aspartic proteinase inhibitor pepstatin A was evaluated (Figures V.3B and V.4). It was found that inclusion of pepstatin A markedly reduced tissue damage (as determined by the LDH assay) after 24 h infection (Figure V.4). Interestingly, in some cases, such as for *C. parapsilosis* AD and *C. parapsilosis* 534638, tissue invasion by the strains was still evident (Figure 3B). At the concentrations used, pepstatin A did not affect *C. parapsilosis* growth or the appearance of the uninfected RHOE.

SAP gene expression

ACT1 gene expression was consistently detected in all samples. Table V.3 presents the mean percentage expression, relative to *ACT1*, of *SAP* genes in planktonic and RHOE infecting *C. parapsilosis* cells, obtained by real time-PCR. All *C. parapsilosis* strains in the planktonic form expressed the three *SAP* genes, with the exception of *SAPP3* for *C. parapsilosis* ATCC 22019^{T}. The *C. parapsilosis* strains studied did exhibit different *SAP* gene expression profiles after RHOE infection. Under these conditions, *SAPP2* gene expression was not detectable for *C. parapsilosis* 513143 and *C. parapsilosis* 491861 (both vaginal strains), whilst *SAPP3* gene expression was not evident for *C. parapsilosis* 513143 (vaginal strain) and *C. parapsilosis* ATCC 22019^{T}. However, there was an apparent elevation of most *SAP* genes in yeast cells infecting the RHOE compared with their planktonic counterparts. The exceptions to this were *SAPP1* with *C. parapsilosis* ATCC 22019^{T} and *C. parapsilosis* 534638, *SAPP2* with *C. parapsilosis* 491861 and *C. parapsilosis* 513143, and *SAPP3* with *C. parapsilosis* ATCC 22019^{T} and *C. parapsilosis* 513143.

Table V.3 Detection of secreted aspartyl proteinase (*SAPP 1-3*) gene expression associated with pathogenesis during planktonic culture and reconstituted human oral epithelium infection (at 24 h) using quantitative real time-polymerase chain reaction

***C. parapsilosis* strain**	**Relative expression of SAP genes***					
	Planktonic cells			**Cells that infected RHOE**		
	SAPP1	*SAPP2*	*SAPP3*	*SAPP1*	*SAPP2*	*SAPP3*
AD	0.097	0.025	0.204	0.318	0.101	0.497
AM2	0.599	0.090	0.546	1.615	0.112	1.064
534638	0.364	0.213	0.142	0.334	0.287	0.408
553877	0.326	0.067	0.021	0.607	0.210	0.092
491861	0.041	0.011	0.208	0.119	ND	0.248
513143	0.039	0.088	0.139	1.426	ND	ND
ATCC 22019^{T}	0.591	0.102	ND	0.066	0.259	ND

ND, indicates that no gene expression was detected.
* The mean arbitrary mRNA transcript levels based upon triplicate measurements, presented as a percentage relative to the respective *ACT1* transcript level.

Discussion

Candida parapsilosis is a normal commensal fungus of humans. Compared with *C. albicans*, relatively few investigations have been carried out to assess the virulence of *C. parapsilosis*, particularly within intact human tissues. The oral epithelium is constantly exposed to microorganisms and is normally an effective barrier against microbial invasion. Thus, the primary aim of this study was to investigate the pattern of colonization and invasion of seven *C. parapsilosis* strains using an *in vitro* RHOE model. This RHOE tissue histologically resembles normal human oral epithelium and is considered to be a good surrogate model to animals for experimental oral candidosis (Schaller *et al.,* 1998; Jayatilake *et al.,* 2005; Malic *et al.,* 2007; Naglik *et al.,* 2008; Lerman *et al.,* 2008).

The results obtained (Table V.2 and Figure V.1) showed that all *C. parapsilosis* strains colonized the RHOE surface. Furthermore, the extent of *C. parapsilosis* colonization was noticeably strain dependent. After 12 h incubation, it was possible to observe clusters of yeasts within the first two layers of the epithelium (Figure V.2); subsequent examination of invasion for each isolate identified significant differences between the 12- and 24-h time-points, indicating progressive tissue damage relating to duration of infection. Compared to the invasiveness of *C. albicans*, described previously (Schaller *et al.,* 2002; Jayatilake *et al.,* 2006; Malic *et al.,* 2007), *C. parapsilosis* has not been considered as highly invasive. However, after 24 h incubation, *C. parapsilosis* induced significant disruption in the tissue architecture of the RHOE (compared with the uninfected controls, Figure V.3A). The degree of tissue damage was further confirmed through the measurement of relative LDH activity (Figure V.4), which was positively correlated with *C. parapsilosis* infection and with histologically observed damage (Figure V.3B). These features are similar to those described in

previous reports (Jayatilake *et al.,* 2006; Gácser *et al.,* 2007) where it was demonstrated that *C. parapsilosis* is able to cause dramatic histopathological tissue changes. The results indicate that the *C. parapsilosis* strains under study produced a variable degree of pathological tissue damage, which was generally higher when compared with *C. albicans* (Schaller *et al.,* 2002; Jayatilake *et al.,* 2006; Malic *et al.,* 2007). The invasive behaviour of *C. albicans* is often attributed to its ability to form hyphae (Bartie *et al.,* 2004; Malic *et al.,* 2007). In the case of the seven *C. parapsilosis* strains investigated, only four were able to form pseudohyphae under the experimental conditions of the study (Table V.2). Despite the extent of damage being strain dependent, this did not appear to correlate with the ability of the organisms to exhibit filamentous growth. Furthermore, when the isolates were grouped according to their clinical origin, there was no obvious association between origin and the ability to colonize or damage the oral epithelium. For instance, the two oral isolates exhibited contrasting behaviour: *C. parapsilosis* AM2 was unable to colonize to a high level or cause damage to the epithelium with associated high LDH levels; *C. parapsilosis* AD colonized to a greater extent and was responsible for a high degree of structural alteration to the oral epithelium with a correspondingly high LDH activity.

Treatment with pepstatin A, an inhibitor of Saps, markedly reduced tissue damage, indicating the potential significance of Saps in this process. Interestingly, and contrast to what has been reported to date (Jayatilake *et al.,* 2006; Gácser *et al.,* 2007), *C. parapsilosis* was able to invade the RHOE and this was not affected by addition of pepstatin A. To explore, in more detail, the possible mechanism of tissue damage and invasion, *SAP* gene expression was assessed using real-time PCR, in all strains of *Candida*, 24 h after infection of RHOE, and expression was compared with that of planktonic cells treat in an identical manner. The production of

extracellular enzymes (particularly Saps), by *C. albicans* has been studied previously (Zaugg *et al.,* 1992; Schaller *et al.,* 1998; Schaller *et al.,* 2000; Malic *et al.,* 2007; Naglik *et al.,* 2008; Lerman *et al.,* 2008), although there still remains doubt over the role of these enzymes in the invasion of epithelial tissue (Schaller *et al.,* 2000; Naglik *et al.,* 2008; Lerman *et al.,* 2008). Overall, the present study showed that expression of *SAP* genes by *C. parapsilosis* was strain dependent, with *SAPP1* being expressed by all infecting isolates. In the case of *SAPP2* and *SAPP3*, two isolates did not express these genes during infection. Interestingly for *SAPP2*, both non-expressing isolates originated from vaginal infections. Furthermore, the pattern of *SAP* gene expression was different between infecting and planktonically cultured *Candida*. Generally, for all *SAPP* genes studied, expression was higher for cells colonizing the tissue, compared with their planktonic equivalents (Table V.3). Given these findings, and the fact that LDH activity in the tissue was reduced as a result of incorporation of pepstatin A, it would appear that Sap enzymes are important instigators of tissue damage by *C. parapsilosis*, but may not be essential for the invasion of the tissue. These results appear to be in agreement with previous studies involving *C. albicans* (Naglik *et al.,* 2008; Lerman *et al.,* 2008).

In summary, this work confirms the effectiveness of RHOE as an *in vitro* model to study *Candida* virulence attributes and broadens the currently limited knowledge on *C. parapsilosis* virulence mechanisms. The CLSM approach has conclusively shown that *C. parapsilosis* is not highly invasive, but can be responsible for significant changes in tissue structure. Furthermore to our knowledge, this is the first report of *SAP* gene expression in *C. parapsilosis* infecting oral epithelium and suggests a role for these enzymes in tissue damage but not candidal invasion.

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References

Bartie KL, Williams DW, Wilson MJ, *et al*., (2004). Differential invasion of *Candida albicans* isolates in an *in vitro* of oral candidosis. *Oral Microbiol Immun* **19**: 293-296.

Bonassoli LA, Bertoli M, Svidzinki TIE (2005). High frequency of *Candida parapsilosis* on the hands of healthy hosts. *J Hosp Infect* **59**: 159-162.

Cairns MT, Church S, Johnston PG, *et al*., (1997). Paraffin-embedded tissue as a source of RNA for gene expression analysis in oral malignancy. *Oral Dis* **3**: 157-161.

Colombo AL, Perfect J, Dinubile M, *et al*., (2003). Global distribution and outcomes for *Candida* species causing invasive candidiasis: results from an international randomized double-blind study of caspofungin versus amphotericin B for the treatment of invasive candidiasis. *Eur J Clin Microbiol Infect Dis* **22**: 470-474.

De Bernardis F, Cassone A, Sturtevant J, *et al*., (1995). Expression of *Candida albicans SAP1* and *SAP2* in experimental vaginitis. *Infect Immun* **63**: 1887-1892.

De Viragh PA, Sanglard D, Togni G, *et al*., (1993). Cloning and sequencing of two *Candida parapsilosis* genes encoding acid proteases. *J Gen Microbiol* **139:** 335**–**342.

Fridkin SK, Kaufman D, Edwards JR, *et al*., (2006). Changing incidence of *Candida* bloodstream infections among NICU patients in the Unite States: 1995-2004. *Pediatrics* **117**: 1680-1687.

Gácser A, Schafer W, Nosanchuck JS, *et al*., (2007). Virulence of *Candida parapsilosis*, *Candida orthopilosis*, and *Candida metapsilosis* in reconstituted human tissue models. *Fungal genet Biol* **44**: 1336-1341.

Haynes K (2001). Virulence in *Candida* species. *Trends Microbiol* **9**: 591-596.

Howlett JA (1976). The infection of rat tongue mucosa in vitro with five species of *Candida*. *J Med Microbiol* **9**: 309-316.

Jayatilake JA, Samaranayake YH, Cheung LK, *et al*., (2006) Quantitative evaluation of tissue invasion by wild type, hyphal and SAP mutants of *Candida albicans*, and non-*albicans Candida* species in reconstituted human oral epithelium. *J Oral Pathol Med* **35**: 484-491.

Jayatilake JA, Samaranayake YH, Samaranayake LP (2005). An ultrastructural and cytochemical study of candidal invasion of reconstituted human oral epithelium. *J Oral Pathol Med* **34**: 240-246.

Kcrmery V, Fric M, Pisarcikova M, *et al*., (2000). Fungemia in neonates: report of 80 cases from seven University hospitals. *Pediatrics* **105**: 913-914.

Lerman U, Morschhauser J (2008). Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*. *Microbiology* 154: 3281-3295.

Malic S, Hill KE, Ralphs JR, *et al*., (2007). Characterization of *Candida albicans* infection of an *in vitro* oral epithelial model using confocal laser scanning microscopy. *Oral Microbiol Immun* **22**:188-194.

Monod M, Togni G, Hube B, *et al*., (1994). Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. *Mol Microbiol* **13**, 357-368.

Naglik JR, Moyes D, Makwana J, *et al*., (2008). Quantitative expression of *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology* 154: 3266-3280

Rossignol T, Logue EM, Keynolds K, *et al*., (2007). Transcriptional response of *Candida parapsilosis* following exposure to Farnesol. *Antimicrob Agents Ch* **51**:2304-2312

Samaranayake LP. Host factor and oral candidosis. In: Samaranayake LP, Macfarlane TW, eds. Oral candidosis. London, UK: Butterworth & Co (Publishers) Ltd, **1990**: 66-103.

Samaranayake YH, Samaranayake LP (2001). Experimental oral candidiasis in animal models. *Clin Microbiol Rev* **14**: 389-429.

Schaller M, Schafer W, Korting HC, *et al*., (1998). Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol Microb* **29**: 605-615.

Schaller M, Korting HC, Schafer W, *et al*., (1999). Secreted aspartic proteinases (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol Microb* **34**: 169-180.

Schaller M, Schackert C, Korting HC, *et al*., (2000). Invasion of *Candida albicans* correlates with expression of secreted aspartic proteinases during experimental infection of human epidermis. *J Invest Dermatol* **114**: 712-717

Schaller M, Mailhammer R, Grassl G, *et al*., (2002). Infection of human oral epithelia with *Candida* species induces cytokine expression correlated to the degree of virulence. *J Invest Dermatol* **118**: 652-657.

Williams DW, Wilson M J, Lewis MAO, *et al*., (1995). Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol* **33**: 2476-2479

Zaugg C, Zepelin MB, Reichard UTZ, *et al*., (1992). Secreted aspartic proteinase family of *Candida tropicalis*. *Infect Immun* **69**: 404-412.

Chapter VI

The role of secreted aspartyl proteinases in *Candida tropicalis* invasion and damage of oral mucosa

The work presented in this chapter was published:

Sónia Silva, Samuel J Hooper, Mariana Henriques, Rosário Oliveira, Joana Azeredo, David D Williams. ***Clinical Microbiology and Infection*** **2010** DOI:10.1111/j.1469-0691.2010.03248

Abstract

Candida virulence attributes include the ability to colonize and invade host tissues, and the secretion of hydrolytic enzymes. Although *Candida albicans* is regarded as the principal fungi causing infections in humans, other species, particularly *Candida tropicalis*, are increasingly recognised as human pathogens. Relatively little is known, however, about the virulence attributes associated with *C. tropicalis*.

The present study aimed to investigate epithelial infection by *C. tropicalis* using a reconstituted human oral epithelium (RHOE) together with confocal laser scanning microscopy (CLSM) and real-time PCR. A comparison of clinical strains was made in terms of tissue colonization, invasion and *C. tropicalis* secreted aspartyl proteinase (*SAPT*) gene expression.

All *C. tropicalis* strains were able to colonize RHOE in a strain-dependent manner. After 12 h of infection, *C. tropicalis* was found to be highly invasive, with extensive tissue damage occurring after 24 h. Real-time PCR of *C. tropicalis SAPT1-4* genes showed that expression was strain-dependent, with *SAPT2-4* transcripts being frequently detected and *SAPT1* rarely detected. Tissue invasion and damage was not inhibited by the presence of pepstatin A. Accordingly, and given that an increase in infection time was not accompanied with an increase in *SAPT* gene expression, it can be suggested that the proteinases are not involved in invasion and damage of RHOE by *C. tropicalis.*

In summary, *C. tropicalis* can be considered highly invasive with the ability to induce significant tissue damage. These features, however, do not appear to be related to specific *SAPT* gene expression.

Keywords: *Candida tropicalis*; candidosis; human epithelium, secreted aspartyl proteinases

Introduction

Many *Candida* species inhabit human mucosal surfaces as harmless commensals. However, these organisms can cause opportunistic infections (candidosis), especially in compromised patients such as those individuals with HIV-infection and diabetes mellitus, and well as in those receiving steroid, broad spectrum antibiotic and cytotoxic drug therapy (Samaranayake, 1990). *Candida albicans* is regarded as the most prevalent species involved in both human colonization and infection (Colombo *et al.,* 2006; Pfaller *et al.,* 2007). Nevertheless, other *Candida* species are increasingly recognized as important agents of human infection (Kcrméry *et al.,* 2002; Hajjey *et al.,* 2004; Ruhnke *et al.,* 2006; Pfaller *et al.,* 2007; Horn *et al.,* 2009). This may reflect the higher level of resistance often exhibited by these species to certain antifungal agents, (González *et al.,* 2008) or an association with modern drug therapies, including the more widespread use of immunosuppressive agents and broad spectrum antibiotics.

Candida tropicalis is a species that is now recognized as an important pathogen of debilitated individuals (Weinberger *et al.,* 2005; Vigouroux *et al.,* 2006; Nucci *et al.,* 2007). However, when compared with the more extensively studied *C. albicans* (De Bernadis *et al.,* 1995; Schaller *et al.,* 1998; Bartie *et al.,* 2004; Jayatilake *et al.,* 2006; Malic *et al.,* 2007), relatively less is known about *C. tropicalis* and its ability to cause human disease (Wingard *et al.,* 1982; Kontoyiannis *et al.,* 2001; Zaugg *et al;* 2001; Arendrup *et al.,* 2002; Al-Fattini *et al.,* 2006; Bizerra *et al.,* 2008). Thus, a greater understanding of the virulence determinants of this species is important because not only would this provide an insight into its pathogenic mechanisms, but also it might promote the development of more effective treatment strategies.

Virulence attributes of *Candida* species include the ability to adhere to host tissue, exhibit morphological alteration, and the secretion of hydrolytic enzymes [*e.g*. phospholipases and secreted aspartyl proteinases (Saps)] (Calderone *et al.,* 2001). The change from a commensal to a pathogen has been related to altered expression of these virulence factors together with a debilitation in host defence mechanisms. Moreover, Saps are considered to be key enzymes that contribute to candidal infection by promoting damage to the host mucosa, thereby facilitating invasion of the organism into the epithelium (Schaller *et al.,* 1999; Zaugg *et al.,* 2001; Naglik *et al.,* 2003). Furthermore, it is known that *C. tropicalis* possesses at least four genes encoding Saps, and these are designated *SAPT1* to *SAPT4* (Togni *et al.,* 1991; Zaugg *et al.,* 2001).

The pathogenesis of mucosal candidosis has been investigated in several studies using animal models, primarily with *C. albicans* (De Bernardis *et al.,* 1995; Samaranayake *et al.,* 2001). In recent years, a commercially available reconstituted human oral epithelium (RHOE) has successfully been used to study the *in vitro* mechanisms of tissue invasion by *C. albicans* (Schaller *et al.,* 1999; Bartie et *al.,* 2004; Jayatilake *et al.,* 2006, 2005; Malic *et al.,* 2007; Lerman *et al.,* 2008; Naglik *et al.,* 2008). Thus, the present study aimed to investigate the pattern of colonization and invasion of RHOE by *C. tropicalis* and to relate it with *SAPs* expression. Specifically, a comparison of clinical strains obtained from different body sites was made in terms of tissue colonization, invasion and *SAP* gene expression using RHOE model, together with confocal laser scanning microscopy (CLSM) and real-time PCR.

Materials and methods

Candida tropicalis strains

A total of six clinical isolates of *C. tropicalis,* originally recovered from the oral cavity (strains AG1 and T2.2), vagina (strains 12 and 75), and urinary tract (strains 519468 and 544123), were used in the present study. A reference strain of *C. tropicalis* from the American Type Culture Collection (ATCC 750^T) was also used. Oral isolates were originally obtained from patients attending the 'Clínica dos Congregados' (Braga, Portugal) and the strains isolated from urinary tract infections were obtained from the 'Hospital de São Marcos' fungal collection (Braga, Portugal). *Candida tropicalis* strains 12 and 75 (recovered from the vaginal tract) were provided by the University of Maringá (Maringá, Brazil). The identification of all isolates was confirmed by PCR-based sequencing using specific primers (ITS1 and ITS4) for the 5.8S subunit gene (Williams *et al.,* 1995).

Isolates were initially cultured on sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) for 48 h at 37°C, followed by culture in yeast nitrogen base medium (BD Diagnostics, Cowley, UK), supplemented with 0.5 % glucose (w/v), for 12 h at 37°C. After incubation, cells were harvested by centrifugation and washed three times with phosphate buffered saline (PBS; pH 7). The yeast cells were then counted using an Improved Neubauer haemocytometer (Marienfield, Land-Könicshofen, Germany) and adjusted to a concentration of 2×10^6 cells ml^{-1}.

In vitro RHOE infection model

Tissue inserts (0.5 cm^2) of commercially available (SkinEthic Laboratories, Nice, France) human oral epithelium, derived from a

squamous carcinoma of the buccal mucosa (human keratinocyte cell line TR 146) and formed on a support membrane, were placed in a new 24-well tissue culture plate. Standardized suspensions of *C. tropicalis* (1 ml containing 2×10^6 cells in MCDB-153 defined medium (Clonetics, San Diego, USA) containing 5 µg ml^{-1} insulin, 1.5 mM $CaCl_2$, 25 µl ml^{-1} gentamicin and 0.4 µg ml^{-1} hydrocortisone) were then added to each RHOE tissue insert. Control samples were inoculated with 1 ml of medium devoid of *Candida* cells. Tissues were subsequently incubated at 37°C in a 5% CO_2 environment with saturated humidity for 12 h and 24 h. In addition, planktonic cultures of *C. tropicalis,* used as controls, were prepared using the same tissue culture medium and incubation conditions, but in the absence of RHOE. These planktonic cultures were subsequently used for standardizing lactate dehydrogenase (LDH) activity and also as controls for *SAP* gene expression studies (24 h).

After incubation, the tissues were rinsed twice with PBS to remove non-adherent *Candida* cells and then bisected, with one half used for CLSM analysis and the other for RNA extraction and real time-PCR studies. The culture medium was also analyzed for *Candida* induced damage using LDH assay.

In separate experiments, the Sap inhibitor, pepstatin A (Sigma, Poole, UK) was added, simultaneously with the *C. tropicalis* inoculum, to the tissue inserts to assess the effect of Saps on the infection process. Briefly, pepstatin A was dissolved in 100% methanol and added directly to the prepared *Candida* inoculum to achieve a final concentration of pepstatin A of 15 µg ml^{-1}. These tissue inserts were examined after 24 h for invasion of *Candida* and LDH production.

Confocal laser scanning microscopy (CLSM)

Colonization and morphological characteristics of *C. tropicalis* on the surface of fresh tissues were assessed using CLSM, following direct labelling of *Candida* cells with 100 µl of concanavalin A lectin conjugated with Alexa 594 (Molecular Probes-Invitrogen, Paisley UK: 25 µg ml^{-1} in PBS) for 20 min at room temperature. Briefly, infected tissue was fixed in 2% (v/v) paraformaldehyde (in ultrapure water) for 24 h at 4°C and embedded in paraffin wax using standard histological techniques. Tissue sections (20 µm) were cut and placed on Histobond+ coated microscope slides (Raymond A Lamb, Eastbourne, UK), de-waxed by processing through xylene, followed by immersion in ethanol and then water. The prepared sections were overlaid with 50 µl of Alexa 594-conjugated concanavalin A lectin (Molecular Probes-Invitrogen, Paisley UK; 25 µg ml^{-1} in PBS) for 20 min at room temperature. For keratinocyte nuclear staining within the RHOE sections, Hoechst 33258 dye (Sigma; 1 µg ml^{-1}) was applied for 30 min at room temperature and then washed with PBS. Tissues were mounted in Vectashield (Vector Laboratories, Peterborough, UK) fade-retarding mountant and then observed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany). The sections were scanned through full depth using appropriate settings for single- and double fluorescence recordings of Hoechst 33258 (laser excitation line 485 nm and emissions detected 410-485 nm) and Alexa 594-conjugated concanavalin A (laser excitation line 546 nm and emissions detected 600-660 nm).

Lactate dehydrogenase (LDH) assay

The release of LDH from the RHOE into the culture medium was used as a measure of epithelial cell damage. The LDH concentration in the

medium from both control and infected tissues was measured at 12 h and 24 h (in both presence and absence of pepstatin A) using the CytoTox-ONE™ kit (Promega, Madison, USA) according to the manufacturer's instructions. A control for LDH activity was also prepared using a replicate preparation of *Candida*, cultured in medium devoid of RHOE. The LDH activity of this control was subtracted from the LDH activity of the tissue infected with yeast. The LDH released during infection with the different *C. tropicalis* strains was then expressed as a relative activity determined against the untreated control tissue. All experiments were performed in duplicate.

Analysis of *SAP* gene expression

RNA extraction

Tissues for RNA extraction were stored at 4°C in 2 ml microtubes containing RNAlater® solution (Ambion, Huntington, UK). Prior to RNA extraction, lysis buffer was prepared by adding 10 µl of ß-mercaptoethanol per ml of RLT buffer (Qiagen, Crawley, UK). Then, 600 µl of lysis buffer and glass beads (0.5 mm diameter, approximately 500 µl) were added to each tissue and these were homogenised twice for 30 s, using a Mini-BeadBeater-8 (Stratech Scientific, Soham, UK). After tissue disruption, the RNeasy Mini Kit (Qiagen) was used for total RNA extraction according to the manufacturer's recommended protocol. Potential DNA contamination was removed by RNase-Free DNase I (Qiagen) treatment. Additionally, RNA was also extracted, following the same approach, from *C. tropicalis* planktonic cells (absence of RHOE) prepared and cultured in the same manner as RHOE infecting strains.

Primer Design

Primers for real-time PCR (RT-PCR) were designed using the Primer3 web-based software (http://frodo.wi.mit.edu\primer3) and these primer sequences are listed in Table VI.1.

Table VI.1 Primers used for real time-PCR analysis of *SAP* and control gene expression

Sequence (5'→ 3')	Primer	Target	PCR product size (bp)
GGAAGATCTGATGTGCCAACTACATTGA	Forward	*SAPT1*	1005
CGTGCGGCCGCTCTACAAAGCCGAGATGTCT	Reverse		
TTCTTCTAGTGGTACCTGGGTCAAAG	Forward	*SAPT2*	762
CATAGATCTCTAAACAATAGTGACATTAGA	Reverse		
ACTTGGATTTCCAGCGAAGA	Forward	*SAPT3*	165
AGCCCTTCCAATGCCTAAAT	Reverse		
GTACTCGAGCTCCTACAACTTCACCTCCT	Forward	*SAPT4*	1130
CATGGATCCCTATGTAAGTGGAAGTATGTT	Reverse		
GACCGAAGCTCCAATGAATC	Forward	*ACT1*	181
AATTGGGACAACGTGGGTAA	Reverse		
CAGGTCCAGGGGTCTTGGTCC	Forward	Human	
GGCAGACCGAGATGAATCCTCA	Reverse	*S14*	143

To verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from ATCC 750^T genomic DNA. Control S14 primers were also used (Cairns *et al.,* 1997) to detect human recombinant DNA.

Synthesis of cDNA

From each sample, 10 µl of extracted RNA was used for cDNA synthesis. First, the RNA was incubated with 5 µl (50 µg ml^{-1}) of oligo-dT

primer (Promega, Southampton, UK) at 70°C for 5 min. Subsequently, RNA was added to a reaction mixture containing RT Buffer [50 mM Tris (pH 8.3), 3 mM $MgCl_2$, 10 U of RNasin Plus RNase inhibitor (Promega)], 20 µM of each deoxynucleoside triphosphate (dNTP) and 200 U of Moloney Murine Leukaemia Virus-Reverse Transcriptase (MMLV-RT, Promega), in a final reaction volume of 50 µl. cDNA synthesis was then performed at 42°C for 1 h. The reaction was stopped by heating for 5 min at 95°C.

Real-time PCR

Real-time PCR was used to determine the relative levels of *SAPT1–4* mRNA transcripts in the RNA samples, with *ACT1* used as a reference housekeeping gene. A 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) was used in accordance with the manufacturer's instructions. Each reaction mixture consisted of the working concentration of Power SYBR® Green PCR master mix (Applied Biosystems), 300 nM forward and reverse primer and 1 µl of cDNA, in a final reaction volume of 20 µl. Negative (water) controls were included in each run. The relative quantification of *SAPT1-4* gene expression was performed by the ΔC_T method, using the control gene (*ACT1*) to normalize the data. Each reaction was performed in triplicate and mean values of relative expression were analysed for each *SAP* gene.

Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) by applying Levene´s test of homogeneity of variance and the Tukey´s multiple-comparisons test, using SPSS (SPSS [Statistical Package for the

Social Sciences], Inc., Chicago, USA). All tests were performed with a confidence level of 95%.

Results

In vitro RHOE infection

Fresh RHOE tissues were used to study surface colonization and candidal morphology after 12 h of incubation (Figure VI.1 and Table VI.2).

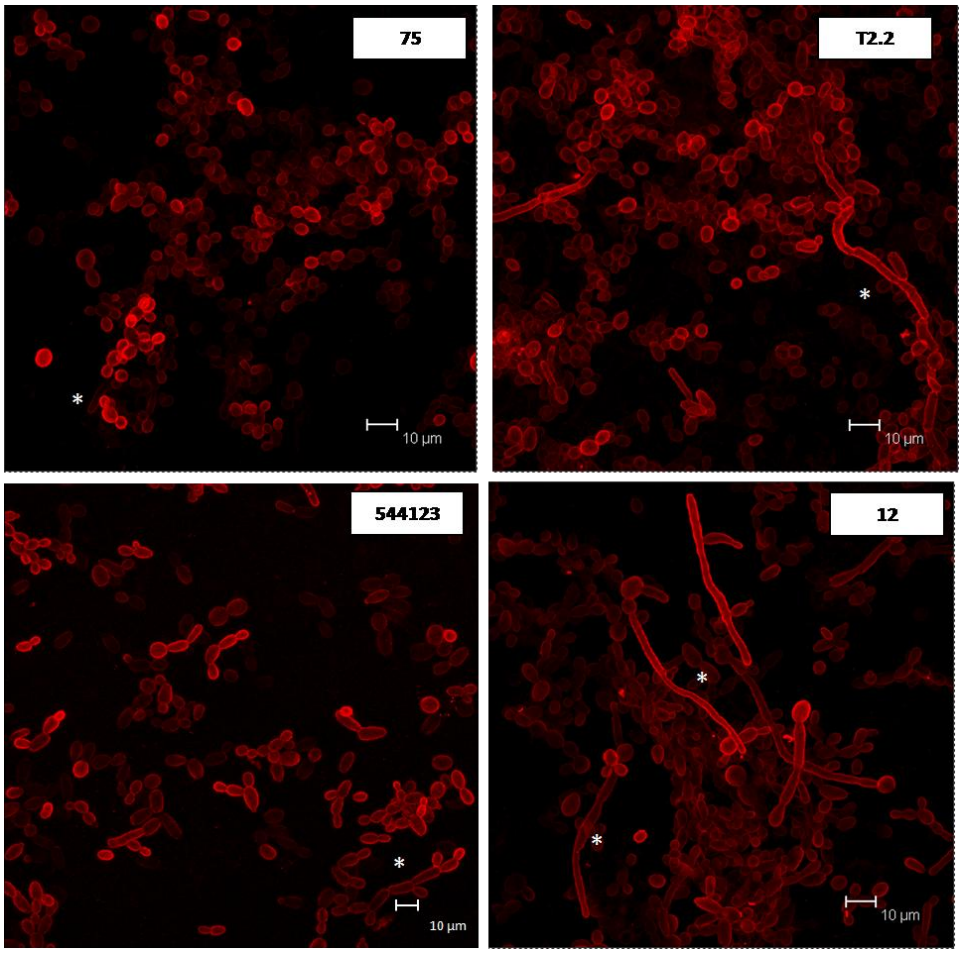

Figure VI.1 Colonization profile of *Candida tropicalis* strains infecting human oral epithelium after 12 h of incubation. *Presence of filamentous forms.

The results showed that all *C. tropicalis* strains were able to colonize the RHOE. However, the extent of colonization was strain-dependent. By

contrast to the other *C. tropicalis* strains, strain 544123 (urinary tract isolate) exhibited a low colonization level.

Interestingly, the different *C. tropicalis* strains exhibited diverse and strain dependent-differences with respect to cell morphologies on the RHOE surface (Figure VI.1 and Table VI.2). *Candida tropicalis* T2.2 and AG1 (oral strains) and *C. tropicalis* 12 (vaginal strain) formed a network of both yeast and filamentous morphology. This contrasted with *C. tropicalis* 519468 (urinary isolate) and *C. tropicalis* ATCC 750^{T}, whit both exhibiting predominantly filamentous growth on the RHOE surface. *Candida tropicalis* 75 (vaginal isolate) and *C. tropicalis* 544123 (urinary tract) strains colonized and grew on the RHOE primarily in yeast form.

Table VI.2 *Candida tropicalis* infection profile of reconstituted human oral epithelium (at 12 and 24 h) as assessed by confocal laser scanning microscopy

***C. tropicalis* strains**	**Origin**	**Surface colonisation** (12 h)	**Strain morphology in presence of tissue** (12h)	**Invasion/integrity of RHOE**	
				12 h	**24 h**
AG1	**Oral**	**+++**	Yeast and filament forms	High/undamaged	High/ damage
T2.2		**+++**	Yeast and filament forms	High/undamaged	High/ damage
519468	**Urinary**	**+++**	Predominantly filament forms	High/undamaged	High/damage
544123		**+**	Predominantly yeast	Low/undamaged	Low/undamaged
12	**Vaginal**	**++**	Yeast and filament forms	High/undamaged	High/damage
75		**++**	Predominantly yeast	High/undamaged	High/damaged
ATCC 750^{T}	**Reference**	**+++**	Predominantly filament forms	High/undamaged	High/undamaged

To assess the invasive capability of *C. tropicalis*, the RHOE tissues were fixed, sectioned, stained and observed by CLSM at 12 h and 24 h after infection (Figures VI.2 and VI.3; Table VI.2).

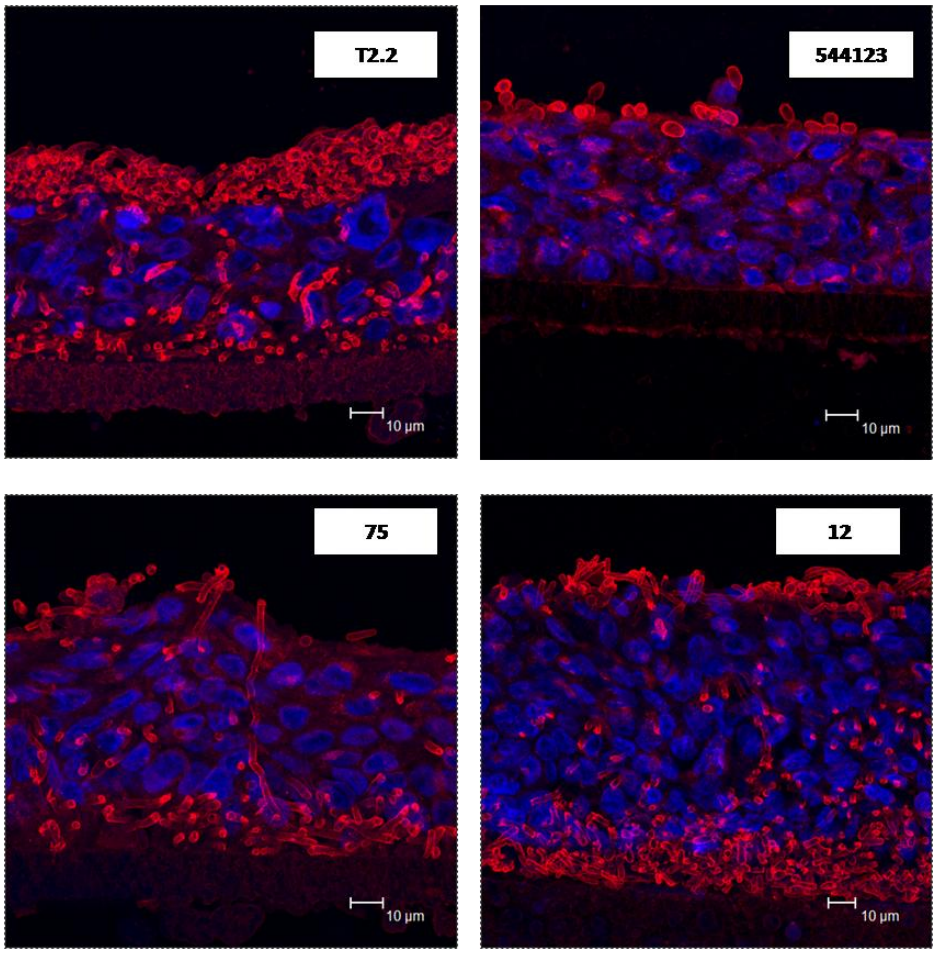

Figure VI.2 Confocal laser scanning microscopy images of *Candida tropicalis* strains infecting human oral epithelium after 12 h of incubation.

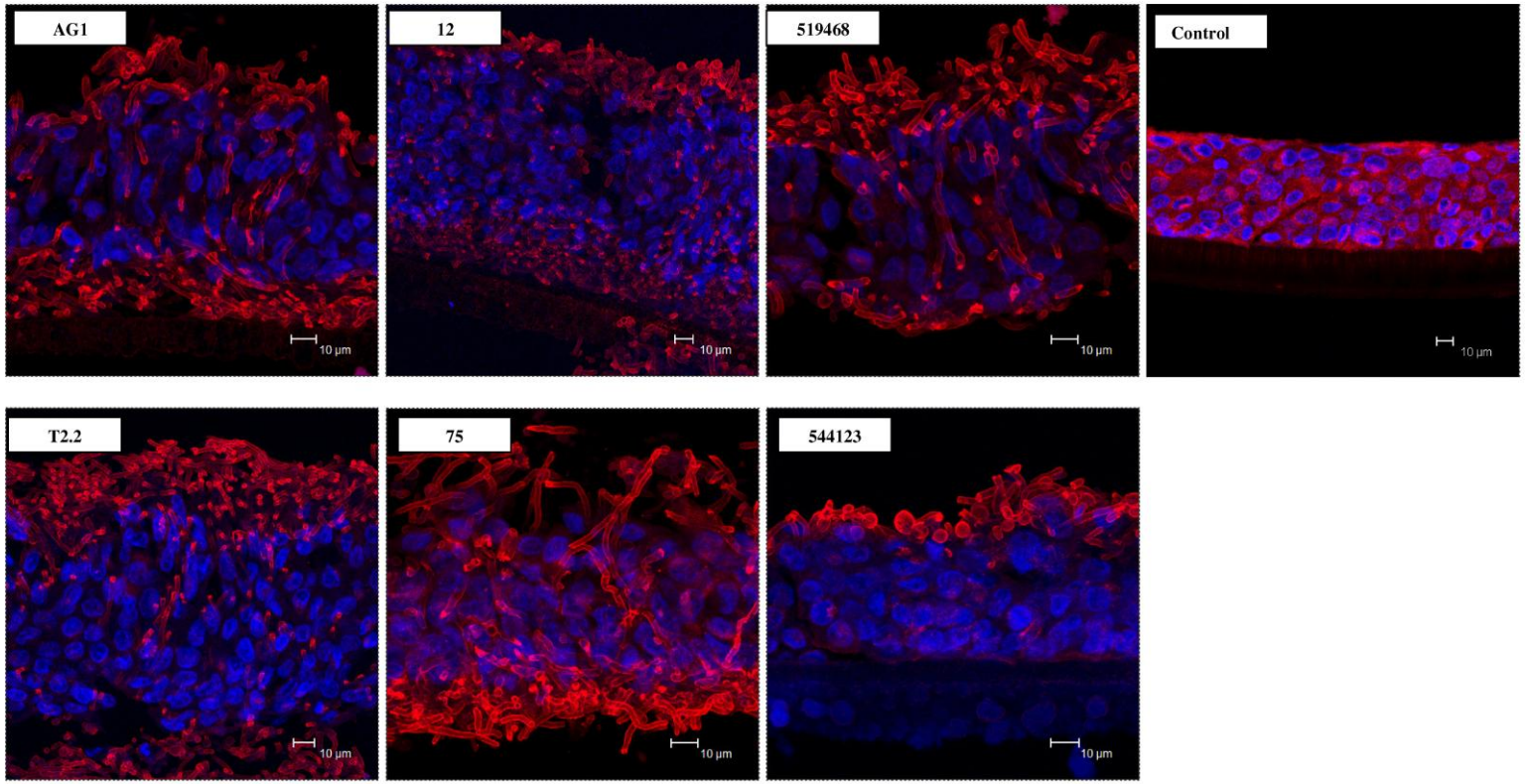

Figure VI.3 Confocal laser scanning microscopy images of *Candida tropicalis* strains infecting human oral epithelium after 24 h of incubation.

Invasion was more extensive after 24 h infection (Figure VI.3) and disruption of the epithelial structure was also more evident. Infection with *C. tropicalis* AG1 and T2.2 (both oral isolates) and *C. tropicalis* 12 (vaginal isolate) induced vacuolization between keratinocytes, promoting a high level of disorganisation of the epithelium structure. Tissue alteration caused by *C. tropicalis* 75 (vaginal isolate) and *C. tropicalis* 519468 (urinary isolate) resulted in the detachment of the superficial keratinocytes layer. Furthermore, the high number of filamentous forms of both strains appeared to be responsible for the detachment of the RHOE from the support membrane. By contrast, changes caused by the non-invasive *C. tropicalis* 544123 (urinary isolate) and by *C. tropicalis* ATCC 750^{T} were limited, despite the latter being able to invade the RHOE.

To quantify tissue damage, the levels of released LDH were determined after 12 h and 24 h of infection (Figure VI.4).

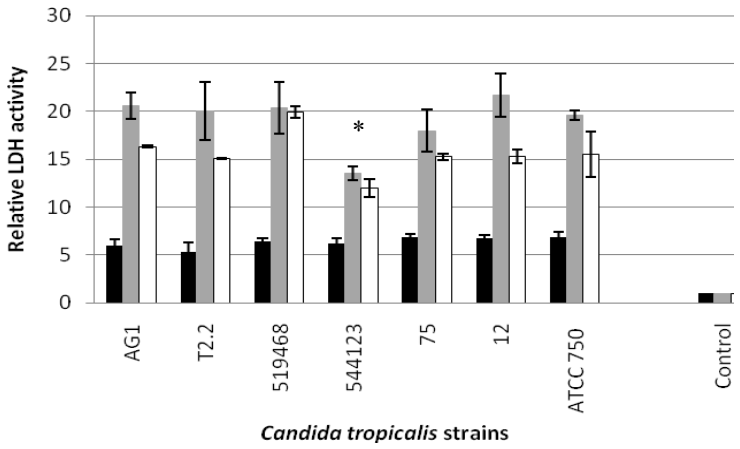

Figure VI.4 Relative LDH activity measured in the human oral epithelium tissue culture supernatant after 12 h (■) and 24 h (■) incubation with different *Candida tropicalis* strains; and in the presence of pepstatin A at 24 h (□). *Strain statistically different from the other strains (P<0.05). Error bars represent standard deviation.

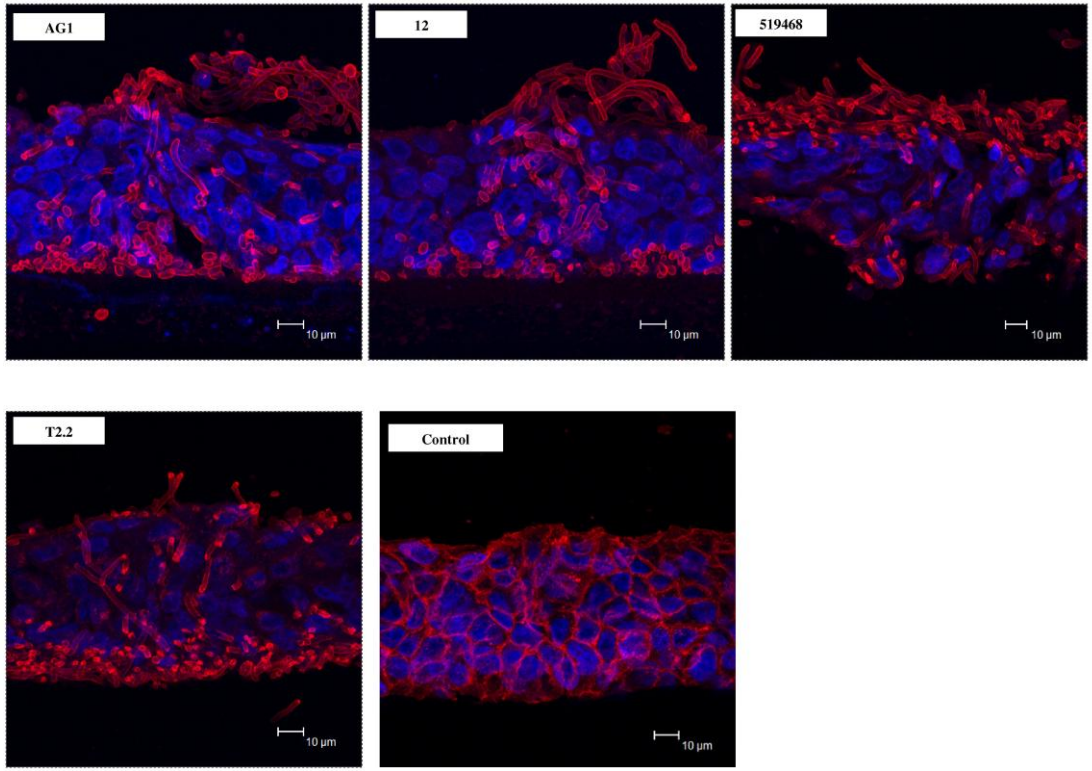

Figure VI.5 Confocal laser scanning microscopy images of *Candida tropicalis* strains infecting human oral epithelium after 24 h in presence of 15 µl ml^{-1} pepstatin A.

Co-culture of tissue with each of the isolates caused a moderate increase in the LDH levels after 12 h incubation compared with uninfected controls. Furthermore, the relative LDH activity was similar for all *C. tropicalis* strains analyzed *(P*>0.05). The levels of LDH markedly increased (approximately 5-fold) with longer infection time (*P*>0.05). The results obtained for LDH activity (Figure VI.4) positively correlated with the extent of tissue damage observed microscopically (Figure VI.3).

Candida tropicalis 544123 caused the least histologically observed damage (not invasive) and also produced the lowest LDH levels, although this was still significantly higher than the uninfected control tissue (Figure VI.4 and Table VI.2).

To provide insight into the mechanism of *C. tropicalis* RHOE invasion and induction of tissue damage, the effect of the specific aspartyl proteinase inhibitor, pepstatin A on the infection process was evaluated (Figures VI.4 and VI.5)

Importantly, invasion of *C. tropicalis* was not inhibited by the presence of pepstatin A (Figure VI.5). Furthermore, the inclusion of pepstatin A did not reduce the level of tissue damage as assessed histologically (Figure VI.5) and by LDH activity (Figure VI.4). At the concentrations used, pepstatin A did not affect *C. tropicalis* colonization or the appearance of the uninfected RHOE.

SAP gene expression

Table VI.3 presents the mean percentage *SAP* gene expression levels relative to *ACT1*, for *C. tropicalis* infecting RHOE for 12 and 24 h, and for planktonic cells (after 24 h incubation using the same conditions, but in the absence of RHOE).

Table VI.3 Detection of secreted aspartyl proteinase (*SAPT1-4*) gene expression associated with pathogenesis during *Candida tropicalis* reconstituted human oral epithelium infection (at 12 h and 24 h) and planktonic culture using quantitative real-time polymerase chain reaction

	Relative expression of SAP genes*											
	Cells that infected RHOE								**Planktonic cells**			
C. tropicalis strains	**12 h**				**24 h**				**24 h**			
	SAPT1	*SAPT2*	*SAPT3*	*SAPT4*	*SAPT1*	*SAPT2*	*SAPT3*	*SAPT4*	*SAPT1*	*SAPT2*	*SAPT3*	*SAPT4*
AG1	ND	0.01±0.00	0.18±0.02	0.28±0.02	ND	ND	0.32±0.15	0.06±0.02	ND	0.02±0.01	1.29±0.42	1.24±0.93
T2.2	ND	0.04±0.02	0.48±0.15	2.70±0.64	ND	ND	0.69±0.27	0.06±0.02	ND	0.03±0.00	1.59±0.16	0.30±0.08
519468	0.03±0.02	0.22±0.14	0.45±0.13	4.57± 0.99	ND	0.22±0.09	ND	0.95±0.54	ND	ND	0.86±0.07	0.16±0.02
544123	ND	0.22±0.03	0.04±0.00	1.58±0.62	ND	0.01±0.00	0.60±0.06	0.02±0.00	ND	0.01±0.01	0.97±0.44	0.10±0.02
12	ND	ND	0.23±0.04	0.01±0.00	ND	0.01±0.01	0.20±0.02	0.05±0.03	ND	ND	0.07±0.03	0.25±0.06
75	ND	ND	0.48±0.06	0.03±0.03	0.03±0.01	0.04±0.02	1.58±0.35	10.07±2.47	0.06±0.01	0.04±0.00	2.27±0.71	9.78±0.41
ATCC 750^{T}	ND	ND	0.76±0.10	0.01±0.07	ND	0.13±0.07	3.92±1.17	0.21±0.07	ND	0.04±0.02	1.07±0.20	0.16±0.13

ND, indicates that no gene expression was detected. *Mean arbitrary messenger RNA transcript levels based upon triplicate measurements, presented as a percentage relative to the respective *ACT1* transcript level.

Real time PCR analysis revealed a wide range of expression profiles of *SAP* genes for the seven *C. tropicalis* strains in the three conditions examined (Table VI.3). In general, *SAPT2-4* transcripts were frequently detected, whereas *SAPT1* was only rarely detected. After 12 and 24 h of incubation, *C. tropicalis* strains exhibited different *SAPT2-4* gene expression profiles.

An increase in the expression of *SAP* genes with a longer incubation time was only evident with *C. tropicalis* ATCC 750^{T} and *C. tropicalis* 75 (*SAPT2-4*), and for *C. tropicalis* AG1, *C. tropicalis* T2.2 and *C. tropicalis* 544123 *(SAPT3*). Furthermore, all *C. tropicalis* strains growing for 24 h in the planktonic form expressed all the *SAPT2-4* genes, with the exception of *SAPT2* for *C. tropicalis* 519468 and *C. tropicalis* 12. Additionally, there was an apparent increase in the expression of most *SAPT* genes for planktonic cells (24 h), when compared to their 24 h RHOE counterparts. For example, with the oral isolate *C. tropicalis* AG1, real time PCR analysis revealed an increased expression of *SAPT3* and *SAPT4* genes, of approximately 4 and 20 times, respectively when compared with RHOE infecting cells.

Discussion

The pathogenesis of mucosal candidosis, particularly those infections caused by *C. albicans,* has been investigated by several authors (Schaller *et al.,* 1998; Bartie *et al.,* 2004; Jayatlake *et al.,* 2006, 2005; Malic *et al.,* 2007; Lerman *et al.,* 2008; Naglik *et al.,* 2008) using RHOE as a successful surrogate model to animals. *Candida tropicalis* has been recognized as an increasingly important pathogen of debilitated individuals (Weininberg *et al.,* 2005; Vigouroux *et al.,* 2006; Nucci *et al.,* 2008), with several studies demonstrating that *C. tropicalis* virulence in mice can be similar to, or indeed higher than *C. albicans* (Wingard *et al.,* 1982; Arendrup *et al.,* 2002). However, compared with *C. albicans*, relatively few investigations have been performed to assess the virulence of *C. tropicalis*, particularly in human tissues (Schaller *et al.,* 2002; Jayatilake *et al.,* 2006, 2008). Thus, the primary aim of the present study was to investigate the pattern of colonization and invasion of RHOE by a variety of *C. tropicalis* strains from different body sites.

The results obtained (Figure VI.1 and Table VI.2) showed that the oral epithelium was successfully colonized by *C. tropicalis*, although the extent of colonization was noticeably strain dependent. The study also revealed that *C. tropicalis* was able to develop filamentous forms on the tissue surface which could promote colonization. Furthermore, CLSM images showed that, similary to *C. albicans* (Jayatilake *et al.,* 2006, 2008; Malic *et al.,* 2007) *C. tropicalis* was also able to invade the RHOE (Figure VI.2). In this regard, invading fungal elements were found to penetrate to the deepest layers of the RHOE within just 12 h of infection (Table VI.2 and Figure VI.2). These observations are not in total agreement with previous reports (Schaller *et al.,* 2002; Jayatilake *et al.,* 2006, 2008), where *C. tropicalis* has been described as a non-invasive species, despite having an

ability to colonize. However, as revealed in the present investigation, strain differences in the capability to invade were apparent.

Subsequent examination of epithelial invasion revealed significant differences between 12- and 24-h infection periods, indicating progressive tissue damage related to the duration of infection. There was induction of significant tissue disruption in the tissue structure (Figure VI.3) compared to uninfected tissues after 24 h infection. The degree of tissue damage was further evaluated quantitatively through the measurement of relative LDH activity (Figure VI.4), and the results obtained positively correlated with *C. tropicalis* infection temporal pattern and histologically observed damage. Interestingly, after 12 h infection, the LDH activities of tissues infected with the different *C. tropicalis* strains were largely similar, implying equivalent degrees of the damage caused. More pronounced differences were found after 24 h of infection, which might reflect a variation in the ability of strains to continue to grow within the epithelium and induce damage after initial invasion. These features were similar to those described in previous reports (Jayatilake *et al.,* 2006, 2008), where it was demonstrated that *Candida dubliniensis*, *Candida glabrata*, *Candida parapsilosis, Candida krusei* and particularly *C. tropicalis*, were causative agents of dramatic histopathological tissue alteration.

Of note, the six strains classified in the present study as 'high invaders' were derived from three different superficial body sites, possibly indicating that strain origin is not important with respect to invade capability. However, further studies involving a greater number of strains, including those from systemic infections, would be of benefit in confirming this view. One strain (*C. tropicalis* 544123) did not produce extensive filamentous forms and, notably, this strain was the only one that was unable to invade the oral epithelium (Figure VI.2, Table VI.2). This result

would support the view that it is the filamentous forms that facilitate tissue invasion.

Secreted aspartyl proteinases (Saps) are considered hydrolytic enzymes contributing to *Candida* infection by promoting damage to the host mucosa, thus facilitating invasion of the epithelium (Zaugg *et al.,* 2001; Naglik *et al.,* 2003). There are at least four known genes encoding for Saps in *C. tropicalis* (Togni *et al.,* 1991; Schaller *et al.,* 1998; Zaugg *et al.,* 2001) and the expression of these *C. tropicalis* genes and the role of Saps in the infection process was assessed in this present study. For all *C. tropicalis* strains, *SAPT* gene expression was assessed using real-time PCR, after 12 h and 24 h of infection (and after 24 h of incubation for equivalent planktonic preparations). Overall, the expression of *SAP* genes by *C. tropicalis* was strain dependent, with *SAPT1* not expressed by the majority of strains, suggesting its limited involvement in invasion and tissue damage. Interestingly, *SAP* gene expression was not significantly different between infecting and planktonic strains of *C. tropicalis*. Furthermore, the effect of the aspartyl proteinase inhibitor, pepstatin A, on the infection process was also evaluated to provide a complementary insight into potential involvement of Saps in *C. tropicalis* invasion and damage to the RHOE. Pepstatin A is a specific inhibitor of acidic proteinases and has been widely used as an inhibitor of *Candida* aspartyl proteinase activity (Schaller *et al.,* 1999; Naglik *et al.,* 2008; Lerman *et al.,* 2008). Interestingly, treatment with pepstatin A did not prevent tissue invasion or apparent histological damage induced by *C. tropicalis* (Figure VI.5). Furthermore, LDH activity was also not reduced by incorporation of pepstatin A, reinforcing the view that the extent of tissue damage was maintained even when Sap activity was inhibited. It would appear therefore that Saps do not play a significant role in tissue invasion and damage caused by *C. tropicalis*. These results appear to be in agreement with recent studies

involving *C. albicans,* which demonstrated that Saps were not required for invasion (Lerman *et al.,* 2008), and damage to RHOE (Naglik *et al.,* 2008).

In summary, the present study confirms the effectiveness of RHOE as an *in vitro* model to study *Candida* virulence attributes and broadens our knowledge on *C. tropicalis* virulence mechanisms. The CLSM approach has conclusively shown that *C. tropicalis* was not only capable of colonizing but could also invade RHOE, leading to significant damage in the tissue structure. Furthermore, to our knowledge, this is the first report of *C. tropicalis SAPT* gene expression during tissue invasion, and the results imply a limited role of these enzymes in *C. tropicalis* invasion and tissue damage.

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References

Al-Fattani MA, Douglas LJ (2006). Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* **55**: 999-1008.

Arendrup M, Horn T, Fridmodt-Moller N (2002). *In vivo* pathogenicity of eight medically relevant *Candida* species in animal model. *Infection* **30**: 286-291.

Bartie KL, Williams DW, Wilson MJ, *et al.,* (2004). Differential invasion of *Candida albicans* isolates in an *in vitro* of oral candidosis. *Oral Microbiol Immunol* **19**: 293-296.

Bizerra FC, Nakamura CV, Poersch C, *et al*., (2008). Characteristics of biofilm formation by *Candida tropicalis* and antifungal resistance. *FEMS Yeast* **8**: 442-450.

Cairns M, Church S, Johnston PG, *et al*., (1997). Paraffin-embedded tissue as a source of RNA for gene expression analysis in oral malignancy. *Oral Dis* **3**: 157-161

Calderone RA, Fonzi AW (2001). Virulence factors of *Candida albicans*. *Trends Microbiol* **9**: 327-335.

Colombo AL, Nucci M, Park BJ, *et al.,* (2006). Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. *J Clin Microbiol* **44**: 2816-2823.

De Bernardis F, Cassone A, Sturtevant J, *et al*., (1995). Expression of *Candida albicans SAP1* and *SAP2* in experimental vaginitis. *Infect Immun* **63**: 1887-1892.

González GM, Elizondo M, Ayala J (2008). Trends in species distribution and susceptibility of bloodstream isolates of *Candida* collected in Monterrey, México, to seven antifungal agents: results of a 3-year (2004 to 2007) surveillance study. *J Clin Microbiol* **46**: 2902-2905.

Hajjeh RA, Sofair AN, Harrison LH, *et al.,* (2004). Incidence of bloodstream infections due *Candida* species and *in vitro* susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J Clin Microbiol* **42**: 1519-1527.

Horn DL, Neofytos D, Anaissie EJ, *et al*., (2009). Epidemiology and outcomes of candidemia in 2019 patients: data from the perspective antifungal therapy alliance registry. *Clin Infect Dis* **48**: 1695-1703.

Jayatilake JA, Samaranayake YH, Cheung LK, *et al*., (2006). Quantitative evaluation of tissue invasion by wild type, hyphal and SAP mutants of *Candida albicans*, and non-*albicans Candida* species in reconstituted human oral epithelium. *J Oral Pathol Med* **35**: 484-491.

Jayatilake JA, Samaranayake YH, Samaranayake LP (2008). A comparative study of candidal invasion in Rabbit tongue explants and reconstituted human oral epithelium. *Mycopathologia* **165**:373-380.

Jayatilake JA, Samaranayake YH, Samaranayake LP (2005). An ultrastructural and cytochemical study of candidal invasion of reconstituted human oral epithelium. *J Oral Pathol Med* **34**: 240-246.

Kontoyiannis DP, Vaziri I, Hanna HA, *et al*., (2001) Risk Factors for *Candida tropicalis* Fungemia in Patients with Cancer. *Clin Infect Dis* **33**:1676–1681.

Krcméry V, Barnes AJ (2002). Non-*albicans Candida* sp. causing fungaemia: pathogenicity and antifungal resistance. *J Hosp Infect* **50**: 243-260.

Lerman U, Morschhauser J (2008). Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*. *Microbiology* **154**: 3281-3295.

Malic S, Hill KE, Ralphs JR, *et al*., (2007). Characterization of *Candida albicans* infection of an *in vitro* oral epithelial model using confocal laser scanning microscopy. *Oral Microbiol Immun* **22**:188-194.

Naglik JR, Challacombe SJ, Hube B (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* **67**: 400-428.

Naglik JR, Moyes D, Makwana J, *et al*., (2008). Quantitative expression of *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology* **154**: 3266-3280.

Nucci M, Colombo AL (2007). Candidemia due to *Candida tropicalis*: clinical, epidemiologic and microbiologic characteristics of 188 episodes occurring in tertiary care hospitals. *Diagn Microbiol Infect Dis* **58**: 77-82.

Pfaller MA, Diekema DJ (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**: 133-163.

Ruhnke M. Epidemiology of *Candida albicans* infections and role of non-*Candida albicans* yeasts. *Curr Drug Targets* 2006; **7**: 495-504.

Samaranayake LP. Host factor and oral candidosis. In: Samaranayake LP, Macfarlane TW, eds. Oral candidosis. London, UK: Butterworth & Co (Publishers) Ltd, **1990**: 66-103.

Samaranayake YH, Samaranayake LP (2001). Experimental oral candidiasis in animal models. *Clin Microbiol Rev* **14**: 389-429.

Schaller M, Korting HC, Schafer W, *et al*., (1999) Secreted aspartic proteinases (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol Microb* **34**: 169-180.

Schaller M, Mailhammer R, Grassl G, *et al*., (2002). Infection of human oral epithelia with *Candida* species induce cytokine expression correlated to the degree of virulence. *J Invest Dermatol* **118**: 652-657.

Schaller M, Schafer W, Korting HC, *et al*., (1998) Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol Microb* **29**: 605-615.

Togni G, Sanglard D, Falcetto R, *et al*., (1991) Isolation and nucleotide sequence of the extracellular acid protease gene (ACP) from the yeast *Candida tropicalis*. *FEBS Lett* **28**: 181-185.

Vigouroux S, Morin O, Moreau P, *et al,*. (2006). Candidemia in patients with hematologic malignancies analysis of 7 years experience in a single center. *Haematologica* **91**: 717-718.

Weinberger M, Leibovici L, Perez S, *et al.,* (2005). Characteristics of candidaemia with *Candida albicans* compared with non-*albicans Candida* species and predictors of mortality. *J Hosp Infe*ct **61**: 146-154.

Williams DW, Wilson M J, Lewis MAO*, et al.,* (1995). Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol* **33**: 2476-2479.

Wingard JR, Dick JD, Merz WG, *et al*., (1982). Differences in virulence of clinical isolates of Candida tropicalis and Candida albicans in mice. *Infection Immunity* **37**: 833-836.

Zaugg C, Zepelin MB, Reichard U, *et al*., (2001) Secreted aspartic proteinase family of *Candida tropicalis*. *Infect Immun* **69**: 405-412.

Chapter VII

Candida glabrata and *Candida albicans* co-infection of an *in vitro* oral epithelium

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Abstract

Candida albicans is regarded as the leading of candidosis. However, *Candida glabrata* has emerged as an important pathogen of the oral mucosa, occurring both singly or in mixed species infections, often with *C. albicans*. Compared with *C. albicans*, little is known about the role of *C. glabrata* in oral infection. The aim of this study was to examine single and mixed species infection of oral epithelium involving *C. glabrata* and establish its ability to invade and damage tissue. A reconstituted human oral epithelium (RHOE) was infected only with *C. glabrata*, or simultaneously with *C. glabrata* and *C. albicans*. The ability of both species to invade the tissue was examined using species specific peptide nucleic acid (PNA) probe hybridisation and confocal laser scanning microscopy (CLSM). Epithelial damage was assessed by measuring lactate dehydrogenase (LDH) activity.

Results showed that *C. glabrata* strains originating from the oral, vaginal and urinary tract were able to colonize the RHOE in a strain dependent manner. Single infection with *C. glabrata* after 12 h, revealed no invasion of the RHOE by any of the *C. glabrata* strains, which contrasted with extensive tissue invasion demonstrated by *C. albicans*. Mixed infection showed that *C. albicans* enhanced the invasiveness of *C. glabrata*, and led to increased LDH release by the RHOE, which paralleled the observed histological damage. This enhanced invasion and increased tissue damage caused by mixed *C. glabrata* and *C. albicans* infections has important clinical significance and highlights the need to identify *Candida* species involved in oral candidosis.

Keywords: candidosis; co-infection; *Candida albicans*; *Candida glabrata*, yeast PNA FISH

Introduction

Candidosis is a frequent opportunistic infection in immunocompromised individuals (Samaranayake, 1990). *Candida albicans* remains the main etiological agent in oral candidosis, accounting for 70% to 80% of isolates from oral mucosal lesions (Pfaller *et al.,* 2000; Trick *et al,* 2002). However, in recent years, *Candida glabrata* has emerged as a notable pathogenic agent of the oral mucosa, either co-infecting with *C. albicans* (Redding *et al.,* 2000, 2002; Li et *al.,* 2007), or occurring in single species infections (Pfaller *et al.,* 2000; Redding *et al.,* 2000, 2002; Li *et al.,* 2007). *Candida glabrata* exhibits significant differences from *C. albicans*, including relative physical size, morphological properties and biochemistry. *Candida glabrata* cells are smaller (1-4 µm) than *C. albicans* yeast (4-6 µm) and have a much narrower spectrum of carbohydrate utilisation (Fidel *et al.,* 1999). Furthermore, in contrast to *C. albicans*, *C. glabrata* is not polymorphic, and only occurs in the yeast form within the oral cavity (Fidel *et al.,* 1999). One possible explanation therefore for the relatively frequent occurrence of co-infection of *C. albicans* and *C. glabrata* is that these significant differences limit the extent of inter-species competition allowing the organisms to occupy similar oral niches. Importantly, *C. glabrata*-associated oropharyngeal candidosis in HIV and cancer patients tends to be more severe and more difficult to treat than infections caused solely by *C. albicans* (Canuto *et al.,* 2000; Redding *et al.,* 2000; 2002).

An understanding of the interactions between these *Candida* species has significance for the development of strategies for oral candidosis management. This is important since, although oral *Candida* infections are not associated with mortality, they are a major cause of morbidity, and trigger chronic pain or discomfort upon mastication, which may limit nutritional intake in immunocompromised or elderly individuals (Redding

et al., 2000; Olmos *et al.,* 2005). In addition, oral carriage of *Candida* has also been proposed to be a predisposing factor for systemic infection in severely immunocompromised patients (Miranda *et al.,* 2009).

Fluorescent *in situ* hybridization (FISH) is rapidly becoming established as a valuable research and diagnostic tool in molecular pathology laboratories, having previously been used to detect and differentiate microorganisms in histological sections (Hayden *et al.,* 2001, 2002; Malic *et al.,* 2009). Peptide nucleic acid (PNA) probes are synthetic equivalents of nucleic acids where the phosphate–sugar polynucleotide backbone has been substituted with a peptide polymer which provides the basis for linking nucleobases. Unlike DNA, the peptide backbone of PNA probes is not charged and this offers greater stability in nucleic acid-probe hybridisation. Furthermore, PNA probes are more resistant to degradation from nuclease and protease activity. Given such advantages, it is not surprising that PNA probes have emerged as valuable tools in the study of infectious diseases and represent a new generation of therapy-directed diagnostics (Oliveira *et al.,* 2002; Guimarães *et al.,* 2007; Malic *et al.,* 2009). To exemplify this, the Yeast Traffic Light PNA FISH^{TM} (YTL, AdvanDx, Inc., Woburn MA) is a newly developed and commercialized PNA probe kit for the rapid detection and distinction of the five most common and clinically relevant *Candida* species (Reller *et al.,* 2007; Shepard *et al.,* 2008).

Previously, a reconstituted human oral epithelium (RHOE) was successfully used to investigate colonization and infection of tissue by *Candida* (Bartie *et al.,* 2004; Jayatilake *et al.,* 2006; Malic *et al.,* 2007; Silva *et al.,* 2009). Coupled with species specific PNA probes and confocal laser scanning microscopy (CLSM), co-infection of RHOE using *C. glabrata* and *C. albicans* can therefore be readily examined.

The aim of this study was to generate single and mixed species infections of RHOE with *C. albicans* and *C. glabrata* and ascertain the role

of *C. glabrata* in the infection using species specific PNA probes, CLSM and lactose dehydrogenase activity (as a measure of tissue damage) in infected tissue.

Materials and methods

Organisms

A total of six clinical isolates of *C. glabrata*, originally recovered from the oral cavity (strains D1 and AE2), vagina (strains 534784 and 585626) and urinary tract (strains 562123 and 513100) were used in this study. A reference strain of *C. glabrata* from the American Type Culture Collection (ATCC 2001) was also included. Oral isolates were obtained from the biofilm group of the Centre of Biological Engineering, Minho University (Braga, Portugal). Strains isolated from urinary and vaginal infections were kindly provided from the culture collection of the Hospital of São Marcos (Braga, Portugal). An oral isolate of *C. albicans* (strain 324LA/94) from the culture collection of Cardiff Dental School (Cardiff, UK) was selected based on its ability to invade RHOE (Malic *et al.,* 2007). The identity of all isolates was initially confirmed by PCR-based sequencing using specific primers (ITS1 and ITS4) for the 5.8S subunit gene (Williams *et al.,* 1995).

Growth conditions

For each experiment, isolates were cultured on sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 48 h at 37°C. Isolates were then subcultured in yeast nitrogen base medium (YNB; BD Diagnostics, Cowley, UK) supplemented with 0.5% glucose (w/v) for 12 h at 37°C. After incubation, cells were harvested by centrifugation and washed three times with Phosphate Buffered Saline (PBS; pH 7). The yeasts were then enumerated using an Improved Neubauer haemocytometer and adjusted to a concentration of 2×10^6 cells ml^{-1} in MCDB-153 defined medium (Clonetics, San Diego, USA).

Reconstituted human oral epithelium (RHOE) infection

To investigate *in vitro* single- and mixed species infection of oral mucosal by *C. glabrata* and *C. albicans* strains, a reconstituted human oral epithelium (RHOE; human keratinocytes derived from a squamous carcinoma of the buccal mucosa, cell line TR 146, SkinEthic Laboratories, Nice, France) model was used. RHOE tissue inserts (0.5 cm^2) were placed in 24-well tissue culture plates with MCDB 153-defined medium containing 5 µg m^{-1} insulin, 1.5 mM $CaCl_2$, 25 µl ml^{-1} gentamicin, and 0.4 µg ml^{-1} hydrocortisone, according to the manufacturer's instructions. For single species infection, 1 ml of standardized suspensions (2×10^6 cells ml^{-1}) of *C. glabrata* or *C. albicans* was placed directly onto the RHOE tissue inserts. For mixed species infection, 500 µl of *C. glabrata* (2×10^6 cells ml^{-1}) was combined with 500 µl of *C. albicans* (2×10^6 cells ml^{-1}) suspension and this preparation placed onto the RHOE tissue inserts. Control samples were inoculated with 1 ml of medium devoid of *Candida*. Infected tissues were incubated at 37°C in a 5% CO_2 environment with saturated humidity for 12 h. After 12 h incubation, the maintenance medium was removed and the RHOE tissues rinsed twice with PBS to remove non-adherent *Candida* cells. The RHOE specimens were then fixed in 2% (v/v, in ultrapure water) paraformaldehyde for 24 h, at 4°C and embedded in paraffin wax using standard histological techniques. Tissue sections (20 µm) were cut and placed onto Histobond+ coated microscope slides (Raymond A Lamb, East Sussex, UK), de-waxed and processed through xylene, ethanol and water.

Peptide nucleic acid fluorescent *in situ* hybridization

The Yeast Traffic Light PNA FISH™ (YTL-PNA FISH) kit (AdvanDx Inc., Woburn MA, USA) was used to examine invasion of the RHOE by *C. glabrata* and *C. albicans*. The *Candida* PNA FISH assay was performed

according to the manufacturer's instructions with some modifications (Reller *et al.,* 2007; Shepard *et al.,* 2008). Sections of RHOE were prepared as described above and then washed briefly in pre-warmed 1× wash solution. Hybridization with PNA probes was performed using 1 drop of *C. albicans*/*C. glabrata* PNA probe mixture and the overlay incubated in the dark in a humidified chamber for 90 min at 55°C. After probe hybridization, excess PNA probe was removed by immersion of the slides into pre-warmed 1× wash solution (55°C) for 30 min. For nuclear staining, keratinocytes within the RHOE sections were counterstained with Hoechst 33258 for 30 min at room temperature, before washing in 1× wash solution. The preparation was then mounted using Vectashield® fade-retarding mountant (Vector Laboratories, Peterborough, UK).

Confocal laser scanner microscopy (CLSM)

Sections hybridized with PNA probes were observed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany). The sections were scanned through the full depth using appropriate settings for single, double or triple channel fluorescence recordings. Fluorescein-labeled PNA was used for detection of *C. albicans* (laser excitation line 492 nm and emissions detected 520 nm), Tamara-labeled PNA was used for detection of *C. glabrata* (laser excitation line 565 nm and emissions detected 580 nm) and Hoechst 33258 (laser excitation line 485 nm and emissions detected 410-485 nm) for nuclear context of keratinocytes. For multi-channel recordings, fluorochromes were scanned sequentially to eliminate spectral overlap between probes. Selected images were presented either as single confocal optical sections or maximum intensity type constructions. *Candida glabrata* and *C. albicans* were identified by red and green coloration respectively, whilst keratinocytes were depicted blue.

Lactate dehydrogenase (LDH) activity

The release of LDH from RHOE into the culture medium was used as a measure of epithelial cell damage. The LDH concentration in the medium from control and infected tissues was determined after 12 h using the CytoTox-ONE™ kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The LDH activity of *Candida* cells in cell culture medium devoid of tissue was subtracted from the LDH activity of the tissue infected with yeasts. The LDH released during single or co-infection with the different *C. glabrata* strains and *C. albicans* was then expressed in relation to the untreated control tissue. All experiments were performed in duplicate.

Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) by applying Levene´s test of homogeneity of variance and the Tukey multiple-comparisons test, using SPSS software (SPSS [Statistical Package for the Social Sciences], Inc., Chicago, USA). All tests were performed with a confidence level of 95%.

Results

Candida glabrata and *Candida albicans* single species RHOE infection

The ability of *C. glabrata* and *C. albicans* strains to colonize and invade human oral epithelium was examined after 12 h of infection using the RHOE model (Figure VII.1 and Table VII.1).

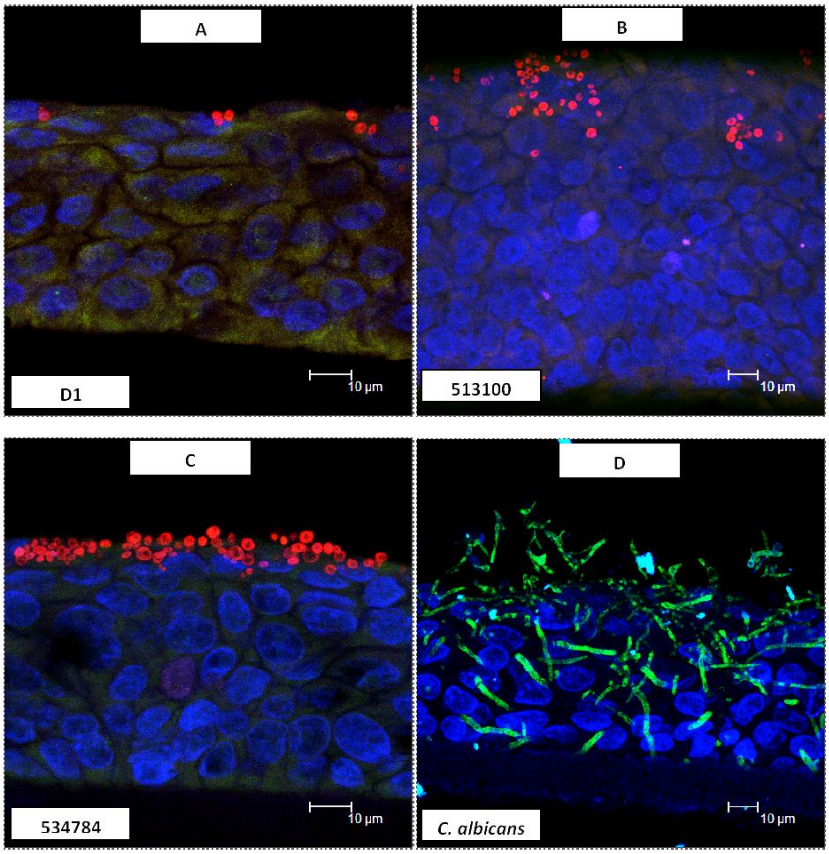

Figure VII.1 Confocal laser scanning microscopy of *Candida* strains after 12 h of RHOE single infection, using YTL PNA FISH™. *C. glabrata* cells are depicted red, *C. albicans* are green, and keratinocytes nuclei are blue. **(A)** *C. glabrata* D1 showing sparse colonization (+) and no invasion; **(B)** *C. glabrata* 513100 showing moderate colonization (++) and moderate invasion; **(C)** *C. glabrata* 534784 showing extensive colonization (+++) and no invasion; (D) *C. albicans* showing extensive colonization (+++) and high invasion.

Results showed that all strains colonized the RHOE surface, although the extent of colonization was highly strain dependent. *Candida glabrata* 534784 and 585626 (both vaginal strains) and *C. albicans* exhibited the highest level of RHOE colonization, whilst *C. glabrata* D1 and AE2 (both oral strains) and 562123 (urinary isolate) colonized the tissue to a lower extent (Figure VII.1).

In general, after 12 h of infection, only clusters of *C. glabrata* yeast were detected at the surface of the keratinocyte layers (Figure VII.1A, C). Furthermore, strains of *C. glabrata* were generally unable to invade the epithelium, although *C. glabrata* 513100 (urinary isolate), was found within the upper (second or third epithelial cell layer) region of the RHOE (Figure VII.1B). In contrast, *C. albicans* showed a greater ability to invade the RHOE with hyphal elements completely penetrating all the epithelial layers (Figure VII.1D). Histological examination of the RHOE after infection with *C. albicans*, demonstrated noticeable changes (Figure VII.1D) compared with tissue infected with *C. glabrata* (Figure VII.1A, B, C). It was evident that *C. albicans* RHOE infection induced superficial detachment of keratinocyte layers (Figure VII.1D).

Table VII.1 *Candida glabrata* and *C. albicans* single and co-infection profile of reconstituted human oral epithelium (at 12h), as assessed using confocal laser scanning microscopy and peptide nucleic acid probe hybridisation

Candida	**Origin**	**Single infection**		**Co-infection**
		Colonization	**Invasion**	**Invasion**
C. glabrata (D1)	Oral	+	None	Moderate
C. glabrata (AE2)		+	None	Moderate
C. glabrata (562123)	Urinary	+	None	Moderate
C. glabrata (513100)		++	Moderate	High
C. glabrata (534784)	Vaginal	+++	None	Moderate
C. glabrata (585626)		+++	None	High
C. *glabrata* (ATCC 2001)	Reference	++	None	High
C. albicans (324LA/94)	Oral	+++	High	-

(+) sparse colinization; (++) moderate colonization; (+++) extensive colonization

In order to quantify tissue damage, the levels of LDH released by epithelial cells after infection were measured (Figure VII.2) and this showed that infection with *C. albicans* produced higher relative levels of LDH activity (P<0.05) compared with *C. glabrata* strains. Furthermore, a moderate but statistically significant increase in relative LDH activity levels was induced by *C. glabrata* strains compared with uninfected controls (P<0.05). Statistical differences with respect to LDH activity released from the RHOE was not evident for *C. glabrata* RHOE infection (P>0.05).

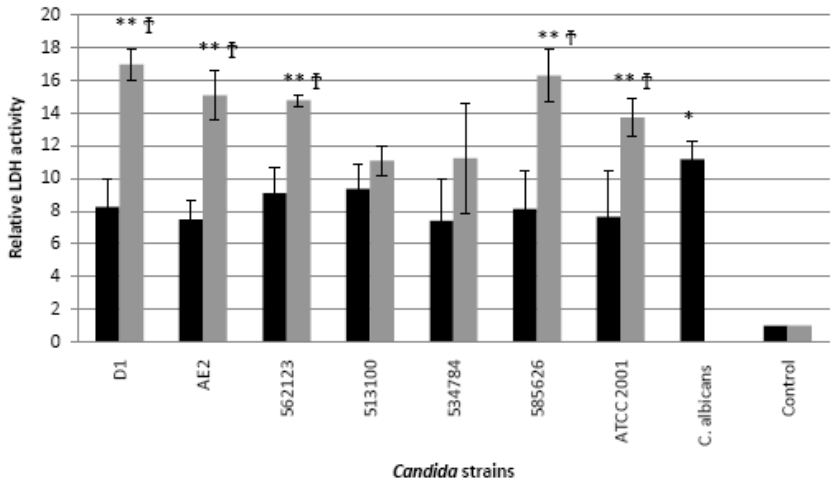

Figure VII. 2 Relative lactate dehydrogenase (LDH) activities measured in the RHOE supernatant after 12 h of single infection (■) and co-infection (■) with *C. glabrata* and *C. albicans*. Control was performed without *Candida* cells. Error bars represent standard deviation. ***** LDH activity of *Candida albicans* single infection is statistically different from *C. glabrata* strains single infections. ****** LDH activity of co-infection is statistically different from the respective single infection by each *C. glabrata* strain. †LDH activity of *C. albicans* single infection is statistically different from co-infection values.

Candida glabrata and *Candida albicans* RHOE mixed species infection

Tissues simultaneously infected with *C. glabrata* and *C. albicans* were subjected to appropriate histological procedures, and the *Candida* species differentiated by hybridization with specific *C. glabrata*/*C. albicans* PNA probes. The relative pattern of infection and ability to invade the RHOE was then observed by CLSM. Contrary to *C. glabrata* single species infection (Figure VII.1 and Table VII.1), when co-infected with *C. albicans*, the majority of *C. glabrata* strains were found to invade the RHOE (Figure VII.3 and Table VII.1).

Yeast of oral isolates (*C. glabrata* D1 and AE2), the urinary isolate (*C. glabrata* 562123) and the vaginal isolate (*C. glabrata* 534784) penetrated to the third epithelial layer of the RHOE (Figure VII.3A). In the case of *C. glabrata* 513100 (urinary isolate), *C. glabrata* 585626 (vaginal isolate) and the reference strain (*C. glabrata* ATCC 2001) penetration to the lowest epithelium layers (Figure VII.3B) was evident. Within the tissue, *C. glabrata* yeast were observed in close proximity to *C. albicans* hyphal elements (Figure VII.4A and C) and localized close to vacuolized tissue portions (Figure VII.4B).

For several strains, the LDH levels released by the RHOE epithelial cells infected by both species increased significantly compared to single species infection (Figure VII.2). Specifically, for tissues co-infected with *C. albicans* and the oral isolates (*C. glabrata* D1 and AE2), the vaginal isolate *C. glabrata* 585626 and the reference strain (*C. glabrata* ATCC 2001) the levels of LDH increased approximately two-fold, whilst a slightly lower (1.5-fold) increase was evident with *C. glabrata* 562123 (urinary isolate). This increase in LDH activity was found to be significant ($P<0.05$).

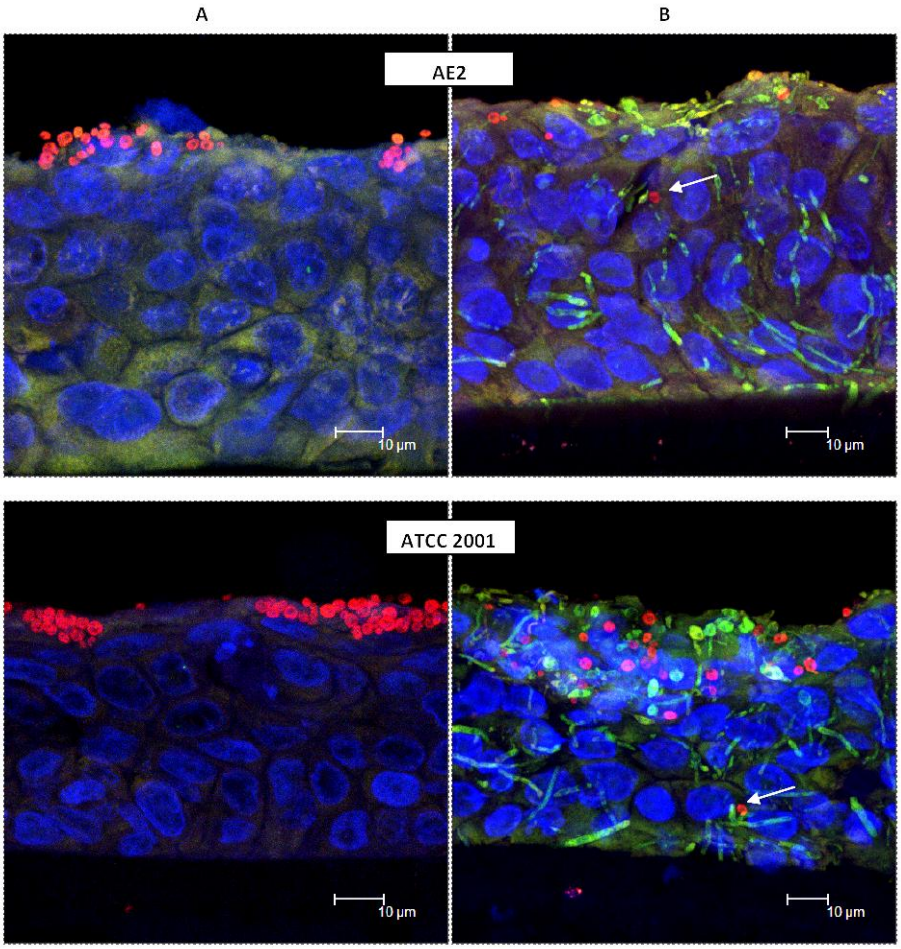

Figure VII.3 Single **(A)** and co-infection **(B)** of *C. glabrata* and *C. albicans* of RHOE after 12 h, assessed by CLSM and YTL PNA FISH™. *Candida glabrata* cells are stained red, *C. albicans* green, and keratinocytes nucleus blue. *Candida glabrata* AE2 presents moderate invasion while *C. glabrata* ATCC 2001 presents high invasion both in the presence of *C. albicans*.

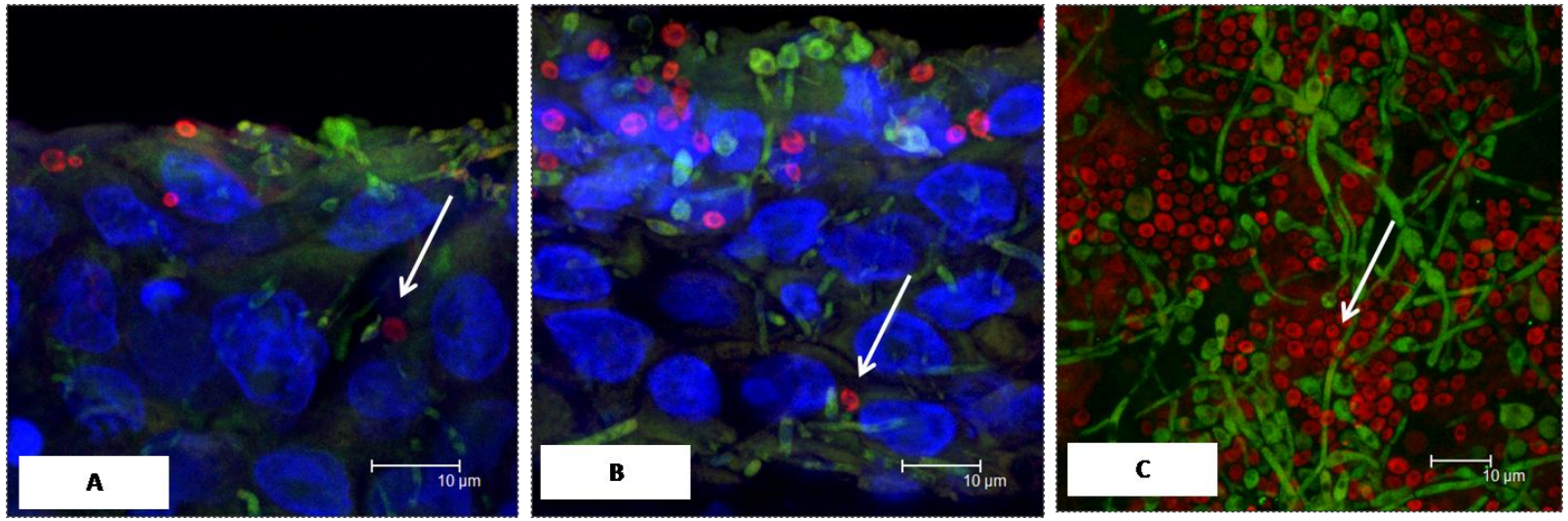

Figure VII.4 *Candida glabrata*/*Candida albicans* co-infection of RHOE after 12 h, assessed by CLSM and YTL PNA FISH™. Panel **A** shows the presence of *C. glabrata* yeast at a vacuolized tissue portion (see arrow), panel **B** illustrates the importance of *C. albicans* hyphal elements on the invasion of *C. glabrata* cells (see arrow) and panel **C** shows the adhesion and co-aggregation of *C. glabrata* cells on hyphal forms of *C. albicans* (see arrow).

However, no statistically differences were found in the LDH activity released by single and co-infection of *C. glabrata* 513100 (urinary isolate) and *C. glabrata* 534784 (vaginal isolate). Furthermore, an increase on the levels of LDH activity was verified between *C. albicans* single infection and co-infection concerning both oral isolates (*C. glabrata* D1 and AE2), one vaginal isolate (*C. glabrata* 585626), one urinary isolate (*C. glabrata* 562123) and the reference strain (*C. glabrata* ATCC 2001) ($P<0.05$). This augment in activity was not found for *C. albicans* co-infecting with one urinary isolate (*C. glabrata* 513100) and one vaginal isolate (*C. glabrata* 534784) ($P>0.05$).

Discussion

Candida species can colonize human oral mucosal surfaces and are frequently present in the oral cavity as harmless commensals (Pfaller *et al.,* 2000; Trick *et al.,* 2002). However these organisms can cause opportunistic infections which are increasingly seen in compromised patients with HIV infection, diabetes mellitus, and in indivudals receiving broad spectrum antibiotics and cytotoxic drug therapies (Samaranayake, 1990; Samaranayake *et al.,* 2002; Rüping *et al.,* 2008).

Although, *C. albicans* is the most frequently encountered pathogen (Pfaller *et al.,* 2000; Trick *et al.,* 2002), several other species, and especially *C. glabrata*, have recently emerged as oral pathogens either found singly or in combination with *C. albicans* (Redding *et al.,* 2000, 2002). *Candida glabrata* is unable to grow in filamentous forms and is therefore only seen as relatively small yeast cells in humans (Fidel *et al.,* 1999). In contrast, *C. albicans* is polymorphic being able to grow as yeast, pseudohyphae and hyphae (Odds, 1998). In terms of tissue invasion, the ability of *C. albicans* to generate hyphae is seen as advantageous as such filamentous growth form enable migration of the fungal element from the point of adherence. Thigmotropic movement is also a property of *Candida* hyphae (Gow, 2009) which could promote invasion of the epithelium (Schaller *et al.,* 2002; Malic *et al.,* 2007; Gow, 2009). The morphological differences and potentially different patterns of growth exhibited by *C. albicans* and *C. glabrata* could allow these species to occupy the same location within the oral cavity with limited competition for space. Furthermore, the fact that *C. albicans* and *C. glabrata* also differ significantly in terms of carbohydrate assimilation profiles (Li *et al.,* 2007) is an additional factor that reduces potential inter-species competition at oral sites, thereby allowing co-existence in many instances.

In recent years, *C. albicans* pathogenesis in mucosal candidosis has been investigated by several authors (Bartie *et al.,* 2004; Jayatilake *et al.,* 2006; Schaller *et al.,* 2006; Malic *et al.,* 2007) using a reconstituted human oral epithelium (RHOE) as a surrogate model to animals. Since mixed infection with both *C. albicans* and *C. glabrata* species has often been reported in the mouth (Redding *et al.,* 2000, 2002), we aimed to examine for the first time the effect of mixed infections in the RHOE model. This in turn would indicate the possible pathogenic significance of mixed infection in the oral cavity.

Initially, the colonization patterns and invasion of RHOE by several *C. glabrata* strains originating from different body sites were examined. The results obtained (Table VII.1, Figure VII.1A-C and Figure VII.3A) showed that the surface of the RHOE was successfully colonized by all *C. glabrata* strains tested, although this was noticeably strain dependent. Furthermore, CLSM revealed that *C. glabrata* were generally unable to invade the RHOE (Figure VII.2). One exception was the urinary isolate *C. glabrata* 513100 which appeared to invade the superficial epithelial layers of the RHOE. These results highlight the diversity of *C. glabrata* strains in colonization and invasiveness capability, and mirror those previously reported for *C. parapsilosis* (Silva *et al.,* 2009), C. tropicalis (Silva *et al.,* 2010) and *C. albicans* (Malic *et al.,* 2007). Schaller *et al.,* (2002) and Jayatilake *et al*., (2006) have both previously classified *C. glabrata* strains as being non-invasive in RHOE infection.

It is important to note that six of the seven strains analysed in this present study and classified as 'non invaders' were sourced from different body sites, possibly indicating that strain origin is not an important aspect in *C. glabrata* colonization and invasion of oral epithelium. For example, the oral isolates (AE2 and D1) exhibited a lower ability to colonize the RHOE, compared with vaginal isolates (534784 and 585626).

Generally, most *C. albicans* isolates infecting the RHOE model exhibit histological signs of hyphal invasion (Odds, 1998; Schaller *et al.,* 2002; Bartie *et al.,* 2004; Malic *et al.,* 2007). This current study also confirmed the high invasiveness of *C. albicans* (Figure VII.1D) and particularly so when compared with *C. glabrata* (Figure VII.1 A-C and Figure VII.3A). In fact, after 12 h of infection, invasion of the RHOE by hyphal elements of *C. albicans* was clearly evident, leading to marked disorganization of RHOE and detachment of superficial layers of the tissue from the insert (Figure VII.1 D); this was in contrast to the lower level of damage caused by *C. glabrata* strains (Figure VII.1 A-C and Figure VII.3 A). The extent of tissue damage was quantitatively assessed by measurement of relative LDH activity (Figure VII.2). The results were in accordance with the extent of *Candida* colonization and tissue damage observed histologically (Figure VII.1). The levels of LDH released during single species infection was highest for *C. albicans* which was expected given the previous histological observations and considering that this species is generally the most virulent *Candida* species (Jayatilake *et al.,* 2006; Schaller *et al.,* 2006).

Interestingly, mixed species infection revealed (Table VII.1 and Figure VII.3B) that *C. albicans* promoted the invasiveness of *C. glabrata.* In fact, six of the seven *C. glabrata* strains classified as 'non-invaders' in single infection studies were found to penetrate the oral epithelium in the presence of *C. albicans*. It is known that *C. albicans* hyphal formation is a critical determinant in invasive pathogenesis especially in comparison with non-*Candida albicans Candida* species, and it has been shown that *C. albicans* with mutations in genes involved in promoting hyphal development were less invasive than wild type strains (Jayatilake *et al.,* 2006).

The exact mechanism of how *C. albicans* might promote *C. glabrata* penetration of the oral epithelium remains unclear. Damage to the

integrity of the epithelial surface caused by the growing tips of the *C. albicans* hyphae (Figure VII.4 A-B) could provide access to lower epithelial layers for *C. glabrata* yeast. It is tempting to speculate that the *C. glabrata* yeast could adhere to *C. albicans* hyphae and be transported in this adhered form to deeper tissue layers. In this context, Ez-Azizi *et al.* (2004) showed the efficient adherence of *C. albicans* to a pre-formed *C. glabrata* biofilm in a catheter model and suggested the possibility of co-aggregation of these two species *in vivo*. RHOE infection studies presented here confirmed adherence of *C. glabrata* cells to hyphal *C. albicans* elements (Figure VII.4C).

The increased LDH activity (Figure VII.2) reported after mixed infection with *C. albicans* and *C. glabrata* supports a finding of enhanced pathogenicity. Of additional concern is that strains of *C. glabrata* often have higher resistance to azoles antifungal agents compared with many other *Candida* species, including *C. albicans* (Redding *et al.,* 2000, 2002; Bagg *et al.,* 2003). In such instances, mixed infection would not only exhibit enhanced pathogenicity, but would also be more problematic to treat. Many diagnostic laboratories are limited in the identification of mixed *Candida* infection, particularly when non differential primary isolation agars such as sabouraud dextrose agar are used. The importance of recognising the occurrence of such mixed infection is however highlighted by the results of this study and represents an area that should be considered by both diagnostic laboratories and clinicians alike.

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References

Bagg J, Sweeney MP, Lewis MA, et al., (2003). High prevalence of non-albicans yeasts and detection of antifungal resistance in the flora of patients with advanced cancer. *Palliat Med* **17**: 477-481.

Bartie K L, Williams DW, Wilson MJ, *et al.,* (2004). Differential invasion of *Candida albicans* isolates in an *in vitro* of oral candidosis. *Oral Microbiol Immunol* **19**: 293-296.

Canuto MM, Gutierrez RF, Ortiz TDV, et al., (2000). Determinants for the development of oropharyngeal colonization or infection by fluconazole-resistant *Candida* strains in HIV-infected patients. *Eur J Clin Microbiol Infect Dis* **19**:593-601.

El-Azizi MA, Starks SE, Khardori NJ (2004). Interactions of *Candida albicans* with other *Candida* spp, and bacteria in the biofilms. *Appl Microbiol* **96**: 1067-73.

Fidel PL, Vazquez JA, Sobel JD (1999). *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin Microbiol Rev* **12**: 80-96.

Gow N (2009). Fungal morphogenesis: some like it hot. *Curr Biol* **19**: 621-629.

Guimarães N, Azevedo NF, Figueiredo C, et al., (2007). Development and application of a novel peptide nucleic acid probe for the specific detection of *Helicobacter pylori* in gastric biopsy. *J Clin Microbiol* **45**: 3089-3094.

Hayden RT, Qian X, Roberts GD, et al., (2001). *In situ* hybridization for the identification of yeast like organisms in tissue section. *Diagn Mol Pathol* **10:**15-23.

Hayden RT, Qian X, Procop GW, et al., (2002). *In situ* hybridization for the identification of filamentous fungi in tissue section. *Diagn Mol Pathol* **11:**119-126.

Jayatilake JA, Samaranayake YH, Cheung LK, *et al.,* (2006). Quantitative evaluation of tissue invasion by wild type, hyphal and SAP mutants of *Candida albicans*, and non-*albicans Candida* species in reconstituted human oral epithelium. *J Oral Pathol Med* **35**: 484-491.

Li L, Redding S, Dongari-Bagtzoglou AD (**2007).** *Candida glabrata*, an emerging oral opportunistic pathogen. *J Dent Res* **86**: 204-215.

Malic S, Hill KE, Hayes A, *et al.,* (2009). Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid (PNA) fluorescent *in situ* hybridization (FISH). *Microbiol* **155**: 2603-2611.

Malic S, Hill KE, Ralphs JR, *et al.,* (2007). Characterization of *Candida albicans* infection of an *in vitro* oral epithelial model using confocal laser scanning microscopy. *Oral Microbiol Immun* **22**:188-194.

Miranda LN, Van der Heijden IM, Costa S, A *et al.,* (2009). *Candida* colonisation as a source for candidaemia. J. Hosp. Infect. **72**: 9-16.

Odds FC. *Candida* and Candidosis, 2n edn. London, Bailliere Tindall, **1998**.

Oliveira K, *Pr*ocop GW, Wilson D., *et al.,* (2002). Rapid identification of *Staphylococcus aureus* directly from blood cultures by fluorescent *in situ* hybridization with peptide nucleic acid probes. *J Clin Microbiol* **40**: 247-251.

Olmos MA, Araya V, Concetti H, *et al*., (2005). Oesophageal candidiasis: clinical and mycological analysis. *Acta Gastroenterol Latinoam* **35**:211-218.

Pfaller M, Diekema ADJ, Jones RN, *et al*., (2000). Trends in antifungal susceptibility of *Candida* spp. isolated from pediatric and adult patients with bloodstream infections: SENTRY Antimicrobial Surveillance Program, 1997 to 2000. *J Clin Microbiol* **40**:852–856.

Redding SW, Kirkpatrick WR, Dib O, *et al*., (2000). The epidemiology of non-*albicans Candida* in oropharyngeal candidiasis in HIV patients. *Spec Care Dentist* **20**:178-181.

Redding SW, Kirkpatrick WR, Coco BJ, *et al*., 2002. *Candida glabrata* oropharyngeal candidiasis in patients receiving radiation treatment for head and neck cancer. *J Clin Microbiol* **40**:1879–1881.

Reller ME, Mallonee AB, Kwiatkowski NP, *et al*., (2007). Use of PNA-FISH for definitive, rapid identification of five common species of *Candida*. *J Clin Microbiol* **45**: 3802-3803.

Rüping MJ, Vehreschild JJ, Cornely OA (2008). Patients at high risk of invasive fungal infections: when and how to treat. *Drugs* **68**: 1941-1962.

Samaranayake LP. Host factor and oral candidosis. In: Samaranayake LP, Macfarlane TW, eds. Oral candidosis. London, UK: Butterworth & Co (Publishers) Ltd, **1990**: 66-103.

Samaranayake LP, Fidel PL, Naglik JR (2002). Fungal infections associated with HIV infections. *Oral Dis* **8**: 151-160.

Schaller M, Zakikhany K, Naglik JR, *et al*., (2006). Models of oral and vaginal candidiasis based on *in vitro* reconstituted human epithelia. *Nat Protoc* **1**: 2767-2773.

Schaller M, Mailhammer R, Grassi G, *et al*., (2002). Infection of human oral epithelia with *Candida* species induces cytokine expression correlated to the degree of virulence. *J Invest Dermatol* **118**: 652-657.

Shepard JR, Addison RM, Alexander BD, *et al*., (2008). Multicenter evaluation of the *C. albicans/C. glabrata* PNA FISH method for simultaneous dual color identification of

Candida albicans and *Candida glabrata* directly from blood culture bottles. *J Clin Microbiol* **46**: 50-55.

Silva S, Henriques M, Oliveira R, *et al*., (2009). Characterization of *Candida parapsilosis* infection of an *in vitro* reconstituted human oral epithelium. *Eur J Oral Sci* **117**: 669-675.

Silva S, Hooper SJ, Henriques M, *et al.,* (2010). The role of secreted aspartyl proteinases in Candida tropicalis invasion and damage of oral mucosa. *Clin Microbiol Inf*. Doi: 10.1111/J. 1469-0691.2010.03248.

Trick WE, Fridkin SK, Edwards JR, *et al*., (2002). National Nosocomial Infections Surveillance System Hospitals. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989-1999. *Clin Infect Dis* **35**:627–630

Williams DW, Wilson MJ, Lewis MA, *et al*., (1995). Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol* **33**: 2476-2479.

Chapter VIII

Concluding remarks and future perspectives

Concluding remarks

Infections caused by *Candida* species have increased greatly over recent years, mainly due to increasing numbers of immunocompromised patients, the ageing population, and the increasing use of indwelling medical devices. *Candida albicans* is the major causative agent of human candidosis, however, and possibly due to new screening techniques other Candida species (NCAC) are increasingly being identified.

The virulence aspects of NCAC species, namely *C. parapsilosis*, *C. tropicalis* and *C. glabrata*, remain poorly understood. As a consequence this work aimed to examine the expression of some of the more relevant virulent factors for NCAC species, such as adhesion and biofilm formation ability, secretion of aspartly proteinases and its role on invasion and damage of an *in vitro* human oral epithelium model.

All NCAC strains (*C. parapsilosis*, *C. tropicalis,* and *C. glabrata*), assessed in this study were able to form biofilm in the presence of a rich culture medium (SDB) and on polystyrene surfaces. The extent of biofilm formation was however both species and strain dependent. Specifically, *C. glabrata* strains were less able to form biofilms than *C. parapsilosis* and *C. tropicalis*. SEM images, indeed, revealed structural and morphological differences for the biofilms of studied NCAC species and strains. However, in general, biofilms of *C. glabrata* presented a more compact biofilm than other species. Concerning, matrix composition, significant and consistent differences were found for the biofilms of different species. Matrices of *C. parapsilosis* biofilms consisted of high amounts of carbohydrate and small amounts of proteins, whilst *C. tropicalis* biofilms were low in both carbohydrate and protein content. Regarding metabolic activity of biofilms, it was possible to conclude that, there were intrinsic differences in terms of metabolic activity amongst biofilms of NCAC strains, which

could have important implications in terms of species relative virulence. The total metabolic activities of biofilms were also strongly species and strain dependent. Furthermore, *C. glabrata* biofilms exhibited the lowest biofilm metabolic activity which was comparable with the lowest value of biofilm or planktonic metabolic activity. Moreover, the absence of a correlation, between cultivable cells number, total biofilm activity and total biofilm biomass, raises the question over which parameter was the most appropriate for *in vitro* assessment of biofilms and their consequent potential clinical significance, stressing out the importance of the use of two or more methodologies together.

In order to better approach and mimic human body conditions, namely those found in urinary catheters (which is the source of severe nosocomial *Candida* infections), adhesion and biofilm formation of NCAC species (urinary isolates) was assessed using silicone and artificial urine (AU). Interestingly, the results showed that all isolates under study were able to adhere to silicone in the presence of AU, but again this was in a species and strain dependent manner. In contrast to the biofilm formation ability studies on SDB and polystyrene, in the presence of AU, *C. glabrata* showed the highest levels of adhesion to silicone when compared with *C. parapsilosis* and *C. tropicalis*. These findings therefore highlighted the importance of varying experimental conditions on biofilm development studies. Additionally, it was also evident that differences in adhesion could not be correlated with cell surface properties since all strains examined presented similar degree of hydrophobicity and exhibited a similar zeta potential. Furthermore, despite the high number of cultivable cells recovered after 72 h of incubation in AU, extensive biofilms were not observed and CLSM showed an absence of extracellular polymeric material for all species. It was therefore shown that these NCAC species were able to adhere and survive on silicone in the presence of urine and the high

levels of *C. glabrata* colonization could be an explanation for the increasing of infections caused by this species in hospitalized and catheterized patients. Furthermore, it is possible to conclude that the virulence factors of NCAC species, such as adhesion and biofilm formation, were strongly dependent not only on the strain type but also the environmental conditions.

In terms of characterization of *C. parapsilosis* and *C. tropicalis* infection of an *in vitro* oral human epithelium (RHOE), it was possible to conclude that all strains from different body sites (oral, vaginal and urinary tract) were able to colonize this epithelium. However, this again was in a strain dependent manner. Strains differences were found in the colonization morphology and in the extent of invasion of the RHOE. Low invasion of RHOE was detected for *C. parapsilosis* strains after 12 h, whereas extensive tissue damage occurred after 24 h when assessed using histological examination and lactate dehydrogenase (LDH) activity determination. Conversely, *C. tropicalis* strains presented higher invasiveness after 12 h of infection, with extensive tissue damage occurring after 24 h. Molecular analysis (real time-PCR) of *SAP* genes, for *C. tropicalis* and *C. parapsilosis* showed that SAP expression was strain dependent for both species. Also, the results suggested that the proteinases were not involved in invasion of RHOE by *C. tropicalis* and *C. parapsilosis*. In addition, pepstatin A was unable to inhibit the invasion of oral epithelium by both species. Furthermore, after 24 h of infection it was evident that a reduction of tissue damage occurred in the presence of pepstatin A in the case of *C. parapsilosis*, but not in case of *C. tropicalis* strains. These findings therefore suggest that Saps could play an important role in tissue damage induced by *C. parapsilosis*.

Regarding, *C. glabrata* and *C. albicans* single and co-infection of RHOE, it was possible to conclude that *C. glabrata* strains originating from

the oral, vaginal and urinary tract were able to colonize the epithelium, again in a strain dependent manner. Single infection with *C. glabrata* after 12 h, generally revealed, no invasion of the RHOE in contrast to tissue invasion caused by *C. albicans, C. tropicalis and C. parapsilosis*. Interestingly, mixed infection (*C. glabrata* and *C. albicans*) showed that *C. albicans* actually enhanced the invasiveness of *C. glabrata*, and led to an increased LDH release by the epithelial cells, which correlated with the observed histological damage. This enhanced invasion and increased tissue damage caused by mixed *C. glabrata* and *C. albicans* infections could have important clinical significance, especially in oral infections where *C. glabrata* (highly resistant to antifungal agents) is often isolated, highlighting the need to a better identification of *Candida* species involved in oral candidosis.

In summary, this work highlights both species and strains differences in terms of the most relevant virulence factors, concerning *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. Furthermore, this work also demonstrated that the virulence factors assessed did not vary with the body origin of the respective strain under study. *Candida glabrata* is the most genetically distinct species compared with *C. tropicalis* and *C. parapsilosis* and this appeared to be reflected by its heterogeneity compared to other to species in terms of the adhesion, biofilm formation, and colonization and invasion of oral epithelium. Since many genes and virulence factors ultimately determine the pathogenicity of opportunistic pathogens and given the increasing incidence of candidosis caused by NCAC species, it remains essential to continue to increase our knowledge base concerning the pathogenic mechanisms of NCAC species. Whilst this study has contributed to this process further work is warranted (see future perspectives). With the advent of new experimental methodology such as CLSM and molecular analysis as utilized in the present study, the further

elucidation of such virulence mechanisms should be an achievable and realistic goal.

Future perspectives

The work described in this thesis provided a useful insight into several aspects of NCAC species pathogenecity, leading to interesting new questions for further research. Some of the suggestions that should be taken into consideration for future investigations are:

a) Assessment of the extension of colonization ability by the same several NCAC strains of reconstituted vaginal epithelium (RHVE). Consequently, stressing the differences among different epithelia and colonization/infection mode.

b) Evaluation of co-infection processes, not only crossing all NCAC species but also using important bacteria, since the great majority of the infections are caused by more than one microorganism.

c) Assessment of the role of other enzymes (phospholipases, lipases and hemolysins) in *Candida* pathogenesis a genomic approach by analyzing the levels of gene expression during tissue interaction and through analysis of the respective deletion mutants.

d) Proteomic approaches could be used with the aim to identify cell wall proteins (CWPs) and other proteins located at the cell surface of NCAC species. Since, CWPs play a crucial role in the initial contacts and interactions between pathogen and host.

e) Adressing the host inflammatory response caused by NCAC species. Since, a more comprehensive study of *Candida* transition from commensalism into a pathogenic state is only possible by addressing the host inflammatory responses. Furthermore, to date, there is still limited knowledge regarding the inflammatory responses to NCAC species.

Summarizing, the recognition of genes and corresponding proteins involved in *Candida*-host interaction as well as the inflammatory response, with the aim to contribute to the elucidation of the underlying molecular mechanisms, and to disclose new potential targets for development of therapeutics against clinically relevant NCAC species fungal pathogens, can be proposed for future work.

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"Se eu fosse compositor

Compunha em vosso louvor

Um hino triunfal

Se eu fosse critico de arte

Havia de declarar-vos

Obra prima à escala mundial

Mas não passo de um homem vulgar

Que tem a sorte de saborear

Esse vosso passo inseguro e o paraíso no vosso olhar"

(Jorge Palma)